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## **Bovine Nebovirus Interacts with a Wide Spectrum of Histo-Blood Group Antigens**

Eun-Hyo Cho, Mahmoud Soliman, Mia Madel Alfajaro, Ji-Yun Kim, Ja-Young Seo, Jun-Gyu Park, Deok-Song Kim, Yeong-Bin Baek, Mun-Il Kang, Sang-Ik Park, et al.

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1 **Bovine Nebovirus Interacts with a Wide Spectrum of**  
2 **Histo-Blood Group Antigens**

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12 Running title: The Wide HBGA Binding Specificity of BNeV

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18 **ABSTRACT** Some viruses within the *Caliciviridae* family initiate their replication cycle by  
19 attachment to cell surface carbohydrate moieties, histo-blood group antigens (HBGAs) and/or  
20 terminal sialic acids (SAs). Although bovine nebovirus (BNeV), one of the enteric  
21 caliciviruses, is an important causative agent of acute gastroenteritis in cattle, its attachment  
22 factors and possibly other cellular receptors remain unknown. Using a comprehensive series  
23 of protein-ligand biochemical assays, we sought to determine whether BNeV recognizes cell  
24 surface HBGAs and/or SAs as attachment factors. It was found that BNeV virus-like particles  
25 (VLPs) bound to A type/H type 2/Le<sup>y</sup> HBGAs expressed in the bovine digestive tract which  
26 are related to HBGAs expressed in humans and other host species, suggesting a wide  
27 spectrum of HBGA recognition by BNeV. BNeV VLPs also bound to large variety of  
28 different bovine and human saliva samples of all ABH and Lewis types, supporting  
29 previously obtained results and suggesting a zoonotic potential of BNeV transmission.  
30 Removal of  $\alpha$ 1,2-linked-fucose and  $\alpha$ 1,3/4-linked-fucose epitopes of target HBGAs by  
31 confirmation-specific enzymes reduced the binding of BNeV VLPs to synthetic HBGAs,  
32 bovine and human saliva, cultured cell lines and bovine small intestine mucosa, further  
33 supporting a wide HBGA binding spectrum of BNeV through recognition of  $\alpha$ 1,2-linked-  
34 fucose and  $\alpha$ 1,3/4-linked-fucose epitopes of targeted HBGAs. However, removal of terminal  
35  $\alpha$ 2,3- and  $\alpha$ 2,6-linked SAs by their specific enzyme had no inhibitory effects on binding of  
36 BNeV VLPs, indicating that BNeV does not use terminal SAs as attachment factors. Further  
37 details of the binding specificity of BNeV remain to be explored.

38 **IMPORTANCE** Enteric caliciviruses such as noroviruses, sapoviruses, and reoviruses are  
39 the most important etiological agents of severe acute gastroenteritis in humans and many  
40 other mammalian host species. They initiate infection by attachment to cell surface  
41 carbohydrate moieties, histo-blood group antigens (HBGAs) and/or terminal sialic acids  
42 (SAs). However, the attachment factor(s) for bovine nebovirus (BNeV), a recently classified  
43 enteric calicivirus genus/type species, remains unexplored. Here, we demonstrate that BNeV  
44 virus-like particles (VLPs) have a wide spectrum of binding to synthetic HBGAs, bovine and  
45 human saliva samples, and bovine duodenal sections. We further discovered that  $\alpha$ 1,2-linked-  
46 fucose and  $\alpha$ 1,3/4-linked-fucose epitopes are essential for binding of BNeV VLPs. However,  
47 BNeV VLPs do not bind to terminal SAs on cell carbohydrates. Continued investigation  
48 regarding the proteinaceous receptor(s) will be necessary for better understanding of the  
49 tropism, pathogenesis and host range of this important viral genus.

50

51 **KEYWORDS** Nebovirus, bovine calicivirus, attachment factor, HBGAs, binding spectrum,  
52 fucose

53           The binding of an infectious virus particle through attachment factors and receptors on  
54 the host cell surface is the essential first step for the viral entry and subsequent replication  
55 therein (1, 2). Generally, attachment factors facilitate the concentration of the incoming virus  
56 particles on the cell surface but do not actively promote entry and mediate signals, whereas  
57 receptors bind the viruses, promote entry, and activate cellular signaling pathways (3). Viral  
58 attachment factors and receptors on host cells comprise a large variety of proteins,  
59 carbohydrates, and lipids with physiological functions unrelated to pathogen interaction (3).  
60 For many viruses, these receptors are glycans linked to either a protein (glycoprotein or  
61 proteoglycan) or a glycolipid (4–6). Glycan-dependent viruses use glycoepitopes as receptors,  
62 binding to negatively charged sialic acids (SAs), sulfated oligosaccharide motifs of  
63 glycosaminoglycan (GAG) chains, or neutral glycoepitopes such as found on histo-blood  
64 group antigens (HBGAs) (6). SAs at the termini or inner portions of glycan chains serve as  
65 receptors for at least ten different virus families, whereas representatives from at least eight  
66 different virus families use GAG chains as receptors (4–7). In contrast, only a few human and  
67 animal viruses within a few families including *Caliciviridae*, *Parvoviridae*, and *Reoviridae*  
68 use HBGAs as receptors (2, 6, 8). The selection of different glycoepitopes as attachment  
69 factors may contribute to virus tropism, pathogenesis, and host specificity (5, 6, 8, 9).

70           Viruses within the family *Caliciviridae* are small, non-enveloped, icosahedral viruses  
71 that possess single-stranded, positive-sense genomic RNA of 7–8 kb in size (10). This family  
72 contains five established genera, *Lagovirus*, *Nebovirus*, *Norovirus*, *Sapovirus*, and *Vesivirus*  
73 (11). Recently, six additional unclassified caliciviruses may represent new genera tentatively  
74 named *Bavovirus* (12, 13), *Nacovirus* (13–15), *Recovirus* (16), *Salovirus* (17), *Sanovirus* (18),  
75 and *Valovirus* (19). Caliciviruses are important etiologic agents in humans and animals,

76 causing a variety of diseases in their respective hosts, such as respiratory disease (feline  
77 calicivirus [FCV]), hemorrhagic disease (rabbit hemorrhagic disease virus [RHDV]), and  
78 gastroenteritis (norovirus [NoV], sapovirus [SaV], and nebovirus [NeV]).

79 Several caliciviruses utilize cell surface carbohydrate moieties, SAs or HBGAs as  
80 attachment factors (2). The initial observation that the *Lagovirus* RHDV uses the H type 2  
81 HBGA as an attachment factor (20) inspired studies to identify similar factors for the other  
82 members of the *Caliciviridae* family (21). These studies showed that different HBGAs are  
83 used as attachment factors for human NoVs (HuNoVs) (21, 22), bovine NoV (23), canine  
84 NoVs (24), and primate enteric caliciviruses within the *Recovirus* genus (25). In contrast,  
85 FCV (26), murine NoV (MNV) (27), and porcine SaV (PSaV) (28) utilize terminal SAs as  
86 attachment factors. Recently, it was observed that some HuNoVs and monkey recoviruses  
87 may also utilize SAs as attachment factors (29, 30). Finally, proteinaceous cellular surface  
88 structures were identified as receptors for a few caliciviruses, such as CD300lf and CD300ld  
89 for MNV (31, 32) and junctional adhesion molecule-1 (JAM-1) for FCV and Hom-1  
90 calicivirus (33–35).

91 HBGAs are complex carbohydrates linked to glycoproteins or glycolipids found in red  
92 blood cells and epithelial cells of the gastrointestinal, genitourinary, and respiratory tracts in a  
93 wide variety of species (2, 8). They can also be secreted as free oligosaccharides into bodily  
94 fluids, such as saliva, intestinal content, milk, and blood (2, 8). The ABH and Lewis HBGAs  
95 are synthesized by the stepwise addition of monosaccharide units to five different types of  
96 precursor: type 1 (Gal $\beta$ -3GlcNAc $\beta$ 1-R), type 2 (Gal $\beta$ -4GlcNAc $\beta$ 1-R), type 3 (Gal $\beta$ -  
97 3GalNAc $\alpha$ 1-R), type 4 (Gal $\beta$ -3GalNAc $\beta$ 1-R), and type 5 (Gal $\beta$ -4Glc $\beta$ 1-Cer) (36). Each step  
98 is catalyzed by specific glycosyltransferases, such as  $\alpha$ -1,2 fucosyltransferase (FUT2),  $\alpha$ -1,3

99 or  $\alpha$ -1,4 fucosyltransferase (FUT3), and two glycosyltransferases (A and B enzymes) (2, 8).  
100 For example, the  $\alpha$ -1,2 fucosyltransferase adds a fucose residue at the  $\alpha$ -1,2 linkage position  
101 of galactose, generating H antigen motifs (2, 8). The addition of N-acetylgalactosamine  
102 (GalNAc) or galactose at the  $\alpha$ -1,3 position of H type chains via A enzyme or B enzyme  
103 respectively results in either A or B antigen (2, 8, 24). The *FUT3* gene, as well as the *FUT4*,  
104 *FUT5*, *FUT6*, *FUT7*, or *FUT9* genes, generate the Lewis antigens by adding a fucose residue  
105 at either the  $\alpha$ -1,3 or  $\alpha$ -1,4 linkage position of the N-acetylglucosamine (GlcNAc) in the type  
106 1 and/or type 2 precursors (2, 8, 24).

107 The genus *Nebovirus* contains one established type species, Newbury-1 virus that  
108 contains Nebraska-like and Newbury1-like clades (37, 38). Recently, two more species in the  
109 genus *Nebovirus* have been identified (39, 40). The reported fecal prevalence of the bovine  
110 nebovirus (BNeV) in calf diarrhea is 5% in Tunisia (41), 4.8% in Brazil (42), 7% in France  
111 (40), 9.1% in Korea (38), 13.1% in Italy (43), and 21.6% in the United States (44). Moreover,  
112 the BNeV prototype strains Newbury1 and Nebraska experimentally induce diarrhea and  
113 small intestinal pathology such as the desquamation of villous epithelial cells and villous  
114 atrophy in gnotobiotic calves (45–47). Despite its significant economic impact on the  
115 livestock industry and status as a pathogen with zoonotic potential, the BNeV life cycle  
116 remains largely unknown, mainly due to a lack of a robust and reproducible in vitro  
117 cultivation systems. Based on the information that either SAs or HBGAs are used as  
118 attachment factors for many caliciviruses, we hypothesized that BNeV might also recognize  
119 either SAs or HBGAs as attachment factors for entry and infection. Therefore, the objective  
120 of this study was to determine the interaction between BNeV virus-like particles (VLPs) and  
121 either SAs or HBGAs using a comprehensive series of BNeV-ligand biochemical assays in

122 synthetic HBGAs, bovine and human saliva samples, cell cultures, and bovine intestinal  
123 tissue sections.



## 124 **RESULTS**

125 **Production and characteristics of BNeV VLPs and hyperimmune antiserum.** The  
126 VLPs produced from the *Spodoptera frugiperda* ovarian (Sf9) cells infected with the  
127 recombinant baculovirus rMA415 had a size of 35-40 nm and appeared empty by electron  
128 microscopy (EM) due to the lack of viral nucleic acids (Fig. 1A). Hyperimmune antiserum  
129 generated from a rabbit immunized three times with purified VLPs of rMA415 by CsCl  
130 density gradient ultracentrifugation detected a specific signal by immunofluorescence in Sf9  
131 cells infected with recombinant baculovirus rMA415 but not in wild-type baculovirus-  
132 infected SF9 cells (Fig. 1B). Western blotting with rMA415 hyperimmune antisera detected a  
133 58-kDa protein as expected and consistent with previous reports on other caliciviruses (48,  
134 49).

135 **Carbohydrate moieties act as attachment factors for BNeV.** To examine whether  
136 carbohydrate moieties are used as attachment factors for BNeV, the carbohydrate moieties  
137 were removed from MDBK cells by pretreatment with sodium periodate ( $\text{NaIO}_4$ ), which is  
138 known to remove the carbohydrate groups without altering cell surface proteins or  
139 membranes (50). The binding of Alexa Fluor 594 (AF594)-labeled BNeV VLPs was slightly  
140 but significantly increased by pretreating the MDBK cells with 1 mM  $\text{NaIO}_4$  but was  
141 markedly decreased with 10 mM  $\text{NaIO}_4$  (Fig. 2A). Pretreatment of cells with  $\text{NaIO}_4$  dose-  
142 dependently decreased the binding of the SA-dependent FCV F9 strain, consistent with the  
143 high sensitivity of SA to periodate treatment (26). HBGA-dependent VLPs from the HuNoV  
144 strain VA387 showed a binding pattern similar to that of BNeV, indicative of binding to  
145 neutral sugars (51, 52). However, 1 or 10 mM  $\text{NaIO}_4$  pretreatment had no inhibitory effect on

146 the binding of the Coxsackievirus B3 (CVB3) strain Nancy, which is known to use decay-  
147 accelerating factor (DAF) as a cellular receptor (7, 53).

148 To precisely quantify the inhibitory effect of NaIO<sub>4</sub> treatment, radioisotope (RI)-labeled  
149 BNeV VLPs and the FCV and CVB3 strains were incubated with the cells pretreated with or  
150 without NaIO<sub>4</sub> (as mentioned above) and then the degree of binding was measured for each  
151 virus by liquid scintillation counting (28). As expected, binding of BNeV VLPs increased to  
152 124% with the 1 mM NaIO<sub>4</sub> treatment and decreased to 55% with the 10 mM NaIO<sub>4</sub>  
153 treatment in comparison with the mock-treated, VLP-inoculated control (Fig. 2B). The  
154 binding of SA-dependent FCV was strongly decreased by both 1 and 10 mM NaIO<sub>4</sub> treatment,  
155 whereas DAF-dependent CVB3 was not influenced by the treatment (Fig. 2B). Taken  
156 together, these results strongly suggest that neutral carbohydrate moieties are involved in the  
157 binding of BNeV VLPs to cells.

158 **Terminal SAs are not recognized by BNeV for attachment.** SAs represent a family  
159 of sugar molecules that are found mostly at the terminal end of carbohydrates and attach to  
160 underlying glycans via  $\alpha$ 2,3-,  $\alpha$ 2,6-, or  $\alpha$ 2,8-linkages (4). Because several caliciviruses,  
161 including FCV, PSaV, and MNV, use terminal SAs as attachment factors (5, 26–28), we  
162 examined whether SAs act as attachment factors for BNeV using 100 mU *Vibrio cholera*  
163 neuraminidase (NA) ml<sup>-1</sup> which cleaves  $\alpha$ 2,3-,  $\alpha$ 2,6- and  $\alpha$ 2,8-linked SAs (26, 28).  
164 Pretreatment of MDBK cells with NA increased the binding of AF594-labeled BNeV VLPs  
165 to cells (Fig. 3A), and the binding of RI-labeled BNeV VLPs to cells increased to 123% of  
166 the levels observed in the mock-treated cells (Fig. 3B). A similar degree of enhanced binding  
167 was observed in the cells treated with HBGA-dependent HuNoV P particles (Fig. 3A).  
168 However, SA-dependent FCV showed a marked reduction in cell binding, whereas with

169 DAF-dependent CVB3 the NA pretreatment had no influence on the degree of binding to  
170 cells (Fig. 3). These results support the notion that BNeV does not use terminal SAs as  
171 attachment factors and that it uses neutral carbohydrate motifs such as HBGAs instead.

172 **Wide binding spectrum of BNeV VLPs to HBGAs.** Using a synthetic HBGA binding  
173 assay, we next determined whether HBGAs could be used as attachment factors for BNeV as  
174 has been described for other viruses elsewhere (28, 54–56). The BNeV VLPs strongly bound  
175 to the immobilized synthetic disaccharide (Fuc $\alpha$ 1,2Gal) (Fig. 4A), a common motif in  
176 HBGAs (57). Moreover, SLe<sup>a</sup>, Le<sup>y</sup>, Le<sup>x</sup>,  $\alpha$ Gal, H2, H1, SLe<sup>x</sup>, H2, and B type immobilized  
177 synthetic HBGA oligosaccharides interacted with the BNeV VLPs (ordered from highest to  
178 lowest binding degree) (Fig. 4A). The recombinant GST-P particles of the HuNoV strain  
179 VA387 and the GST-VP8\* proteins of the human rotavirus G11P[25] Dhaka6 and bovine  
180 rotavirus G6P[5] WC3 strains bound to their corresponding HBGA types, whereas the  
181 supernatant of wild-type baculovirus-infected Sf9 cells and GST had no binding affinity to  
182 any HBGA (Fig. 4A). These results indicate that BNeV VLPs recognize a wide spectrum of  
183 HBGAs.

184 **HBGA-binding moiety for BNeV VLPs.** To identify HBGA-binding epitopes, we  
185 examined whether removal of each putative epitope from synthetic HBGAs by pretreatment  
186 with  $\alpha$ 1,2-L-fucosidase,  $\alpha$ 1,3/4-L-fucosidase,  $\alpha$ -galactosidase, or  $\alpha$ N-acetylgalactosaminidase  
187 was able to decrease the HBGA binding of BNeV VLPs. The results showed that treatment  
188 with  $\alpha$ 1,2-L-fucosidase, which removes the  $\alpha$ -1,2-linked-fucose from galactose, significantly  
189 decreased the binding of BNeV VLPs to the H type disaccharide, H1 and H2 types, and Le<sup>y</sup>  
190 carrying  $\alpha$ 1,2-linked-fucose epitope (Fig. 4B), suggesting that BNeV VLPs recognize the  
191  $\alpha$ 1,2-linked-fucose as an epitope. In contrast, pretreatment with  $\alpha$ 1,3/4-L-fucosidase resulted

192 in a significant reduction in the binding of BNeV VLPs to SLe<sup>a</sup>, Le<sup>x</sup>, SLe<sup>x</sup>, and Le<sup>y</sup> that all  
193 harbor the  $\alpha$ 1,3/4-linked-fucose epitope (Fig. 4B), confirming binding specificity for this  
194 epitope. An almost complete reduction in the binding of BNeV VLPs to Le<sup>y</sup>, containing both  
195  $\alpha$ 1,2- and  $\alpha$ 1,3/4-linked-fucose epitopes, was observed by combined pretreatment of synthetic  
196 Le<sup>y</sup> with both enzymes,  $\alpha$ 1,2-L-fucosidase and  $\alpha$ 1,3/4-L-fucosidase, supporting the above  
197 results that BNeV VLPs recognize both  $\alpha$ 1,2-linked-fucose- and  $\alpha$ 1,3/4-linked-fucose.  
198 Pretreatment of the Gal $\alpha$ 3Gal $\beta$ 4GlcNAc $\beta$  HBGA with  $\alpha$ -galactosidase that cleaves the  $\alpha$ Gal  
199 epitope in Gal $\alpha$ 3Gal $\beta$ 4GlcNAc $\beta$  HBGA had no inhibitory effect on the binding of BNeV  
200 VLPs to synthetic Gal $\alpha$ 3Gal $\beta$ 4GlcNAc $\beta$  HBGA (Fig. 4B). However, it significantly reduced  
201 the binding of the control GST-VP8\* protein of the  $\alpha$ Gal-dependent bovine rotavirus P[5]  
202 WC3 strain to the synthetic Gal $\alpha$ 3Gal $\beta$ 4GlcNAc $\beta$  HBGA (Fig. 5), suggesting that the  $\alpha$ Gal  
203 epitope is not recognized by the BNeV VLPs. Altogether, these results suggest that BNeV  
204 VLPs recognize fucose residues in  $\alpha$ 1,2- and  $\alpha$ 1,3/4-linkages.

205 **Saliva binding profile of BNeV VLPs.** Because saliva contains mucins carrying  
206 HBGAs that are similar to those expressed in the small intestine (2, 8), a saliva-binding assay  
207 was performed with the BNeV VLPs, the GST-P particles of the HuNoV strain VA387, and  
208 the GST-VP8\* proteins of the bovine rotavirus P[5] strain WC3 and human rotavirus P[25]  
209 strain Dhaka6 using bovine and human saliva samples, as described elsewhere (55, 56, 58).  
210 Prior to determining the binding affinity of BNeV VLPs in the bovine and human saliva  
211 samples, the expression levels of each HBGA in the bovine and human saliva samples were  
212 examined by enzyme immunoassays as described elsewhere (51, 52, 56). Consistent with  
213 previous HBGA phenotyping results for bovine gastrointestinal mucosa (23), the bovine  
214 saliva samples contained individually varying levels of A type, H type 2, Le<sup>y</sup> and

215 Gal $\alpha$ 3Gal $\beta$ 4GlcNAc $\beta$  HBGAs and were largely divided into two ABO blood types, H+/A-  
216 /B- and H+/A+/B- (Fig. 6A). In addition, the human saliva samples also had variable levels  
217 of HBGAs in accordance with ABO and Lewis types (Fig. 6B).

218 Subsequently, the binding of BNeV VLPs to HBGAs in the bovine and human saliva  
219 samples was examined. The BNeV VLPs bound to HBGAs in the bovine saliva samples  
220 regardless of the contents of individual HBGAs (Fig. 6A). As a positive control, the  
221 recombinant GST-VP8\* protein of the bovine rotavirus strain WC3 bound to bovine saliva  
222 samples (Fig. 6A). An analogous result was obtained for binding to a range of human saliva  
223 samples, regardless of the contents of individual HBGAs (Fig. 6B). As controls, the  
224 recombinant GST-VP8\* protein of the human rotavirus strain Dhaka6 preferentially bound to  
225 human saliva samples rich in A type HBGA (Fig. 7A), whereas the recombinant GST-P  
226 particles of the HuNoV strain VA387 showed preferential binding to human saliva samples  
227 rich in A and B types of HBGAs (Fig. 7B). All of these data are consistent with the  
228 conclusion that a broad range of HBGAs is recognized by BNeV VLPs.

229 **Binding epitopes for BNeV VLPs in the saliva samples.** To identify binding epitopes  
230 in saliva HBGAs, the effect of pretreating the saliva samples with  $\alpha$ 1,2-L-fucosidase,  $\alpha$ 1,3/4-  
231 L-fucosidase,  $\alpha$ -galactosidase, or  $\alpha$ N-acetylgalactosaminidase, either individually or in  
232 combination, was examined. Four bovine saliva samples were selected, two of which  
233 represented the H+/A+/B- type (samples 2 and 4) and two of which represented the H+/A-/B-  
234 type (samples 6 and 10). As each bovine saliva sample expressed different levels of A type, H  
235 type 2, Le<sup>y</sup> type, and Gal $\alpha$ 3Gal $\beta$ 4GlcNAc $\beta$ , pretreatment with individual enzymes reduced  
236 the binding of BNeV to each saliva sample only mildly (Fig. 8A). However, pretreatment of  
237 saliva samples with a mixture of  $\alpha$ 1,2-L-fucosidase and  $\alpha$ 1,3/4-L-fucosidase resulted in

238 greater inhibition (Fig. 8A). In addition, pretreatment of the bovine saliva samples with  $\alpha$ -  
239 galactosidase and  $\alpha$ N-acetylgalactosaminidase did not inhibit the binding of BNeV VLPs to  
240 any of the selected bovine saliva samples (Fig. 8A), supporting the interpretation that the  
241  $\alpha$ Gal and GalNAc epitopes are not recognized by the BNeV VLPs. To assess the function of  
242  $\alpha$ -galactosidase, the inhibitory effect of  $\alpha$ -galactosidase on the binding of the GST-VP8\*  
243 protein of  $\alpha$ Gal-dependent bovine rotavirus P[5] strain WC3 to each of the bovine saliva  
244 samples was examined. Pretreatment of the bovine saliva samples with  $\alpha$ -galactosidase  
245 reduced the binding with the control GST-VP8\* protein of the  $\alpha$ Gal-dependent bovine  
246 rotavirus P[5] strain WC3 (Fig. 8B).

247 Each human saliva sample used in this study had different ABH and Lewis antigens  
248 depending on the individual (Fig. 6B). Among these samples, six samples representing A  
249 (samples 11, 40, and 70) or H (samples 49, 83, and 95) ABH types were selected. Regardless  
250 of the ABH types, pretreatment of each saliva sample with a mixture of  $\alpha$ 1,2-L-fucosidase  
251 and  $\alpha$ 1,3/4-L-fucosidase inhibited the BNeV binding significantly more than that with either  
252  $\alpha$ 1,2-L-fucosidase or  $\alpha$ 1,3/4-L-fucosidase individually (Fig. 8C). As expected, pretreatment  
253 of the human saliva samples with  $\alpha$ -galactosidase or  $\alpha$ N-acetylgalactosaminidase had no  
254 influence on BNeV binding (Fig. 8C). These results confirmed the wide HBGA-binding  
255 spectrum of BNeV VLPs through recognition of  $\alpha$ 1,2-linked-fucose and  $\alpha$ 1,3/4-linked-fucose  
256 epitopes.

257 **Expression of BNeV-binding HBGA epitopes in cell lines.** To further define the  
258 BNeV-binding HBGA epitopes, the expression level of the different HBGAs was examined  
259 in several cell lines using antibodies specific for each HBGA. The bovine kidney epithelial  
260 MDBK, porcine kidney epithelial LLC-PK, canine kidney epithelial MDCK, and feline

261 kidney CRFK cells solely expressed Gal $\alpha$ 3Gal $\beta$ 4GlcNAc $\beta$  HBGA. Human colorectal  
262 adenocarcinoma Caco-2 cells expressed H types 1 and 2, Le<sup>a</sup>, Le<sup>x</sup>, Le<sup>b</sup>, and Le<sup>y</sup> HBGAs,  
263 whilst human embryonic kidney epithelial 293T cells did not express any of the HBGAs  
264 examined. Among these cell lines, MDBK cells were selected to check whether  $\alpha$ Gal is  
265 recognized as an attachment factor for BNeV binding because of sole expression of the  $\alpha$ Gal-  
266 epitope carrying Gal $\alpha$ 3Gal $\beta$ 4GlcNAc $\beta$  HBGA on the cell surface, whereas Caco-2 cells were  
267 selected to check whether  $\alpha$ 1,2-fucose and  $\alpha$ 1,3/4-fucose epitopes are used as BNeV  
268 attachment factors because of expression of multiple HBGAs containing  $\alpha$ 1,2-fucose and  
269  $\alpha$ 1,3/4-fucose epitopes on the cell surface HBGAs. The binding of AF594-labeled BNeV  
270 VLPs to each cell line was then examined both before and after removal of all of the  
271 corresponding HBGAs expressed in each cell line by pretreatment with  $\alpha$ 1,2-L-fucosidase,  
272  $\alpha$ 1,3/4-L-fucosidase,  $\alpha$ -galactosidase, or  $\alpha$ N-acetylgalactosaminidase, either individually or in  
273 combination. As expected, removal of  $\alpha$ Gal from Gal $\alpha$ 3Gal $\beta$ 4GlcNAc $\beta$  HBGA by  
274 pretreatment with  $\alpha$ -galactosidase had no inhibitory effect on BNeV binding to the MDBK  
275 cells, supporting the conclusion that Gal $\alpha$ 3Gal $\beta$ 4GlcNAc $\beta$  HBGA is not used for BNeV  
276 attachment (Fig. 9A). To confirm the efficacy of  $\alpha$ 1,2-L-fucosidase,  $\alpha$ 1,3/4-L-fucosidase, and  
277  $\alpha$ -galactosidase, MDBK and Caco-2 cells were pretreated with each enzyme and then the  
278 samples were checked for efficient removal of fucose by using *Ulex Europaeus* agglutinin 1  
279 (UEA-1) that detects fucose residues (59) or an antibody specific to  $\alpha$ Gal. The pretreatment  
280 of cells with  $\alpha$ -galactosidase markedly removed  $\alpha$ Gal residue from MDBK cells, whereas a  
281 mixture of  $\alpha$ 1,2-L-fucosidase and  $\alpha$ 1,3/4-L-fucosidase significantly removed fucose residues  
282 from Caco-2 cells (data not shown). Interestingly, pretreatment of MDBK cells with each  
283 fucosidase significantly decreased BNeV VLP binding (Fig. 9A). These effects became more  
284 apparent when the cells were pretreated with the mixture (Fig. 9A) even though the cells did

285 not appear to express fucosylated HBGAs. This suggests that  $\alpha$ 1,2- and  $\alpha$ 1,3/4-linked fucose  
286 residues not detected by these reagents nonetheless support the binding of BNeV VLPs to  
287 MDBK cells. Pretreatment of Caco-2 cells with either  $\alpha$ 1,2-L-fucosidase or  $\alpha$ 1,3/4-L-  
288 fucosidase also reduced the binding of BNeV VLPs (Fig. 9B). The reduction was much  
289 enhanced by pretreatment of the Caco-2 cells with the  $\alpha$ 1,2-L-fucosidase and  $\alpha$ 1,3/4-L-  
290 fucosidase mixture (Fig. 9B), indicating that BNeV VLP binding involved the  $\alpha$ 1,2-linked-  
291 fucose and  $\alpha$ 1,3/4-linked-fucose epitopes of HBGAs.

292 **BNeV attachment to CHO cells with expression of H type 2.** CHO cells do not  
293 express any HBGA on the cell surface due to the lack of  $\alpha$ 1,2-fucosyltransferase activity and  
294 of either the A or B histo-blood group enzymes (60). Whether the above-described binding  
295 between BNeV VLPs and HBGAs was similar to that found in parental CHO or transfectant  
296 CHO cells, parent and transfectant CHO cells expressing H type 2, A type, B type, or  
297 Gal $\alpha$ 3Gal $\beta$ 4GlcNAc $\beta$  HBGAs were examined. After the expression of each target HBGA in  
298 parental CHO and transfectant CHO cells as confirmed (Fig. 10A), binding of BNeV VLPs to  
299 parental and transfectant CHO cells was analyzed. Compared with parental CHO cells (H-  
300 /A-/B-), to which AF594-labeled BNeV VLPs failed to attach, binding of BNeV VLPs was  
301 very prominent in CHO cells expressing H type 2 HBGA (H+/A-/B-) (Fig. 10B). It was also  
302 detected, albeit less extensively, with A (H+/A+/B-) or B (H+/A-/B+) types (Fig. 10B). As  
303 expected, CHO cells expressing Gal $\alpha$ 3Gal $\beta$ 4GlcNAc $\beta$  HBGA had no BNeV VLP binding  
304 (Fig. 10B). Taken together, these findings support the overall conclusion that BNeV VLPs  
305 have a wide HBGA binding spectrum through specific reorganization of  $\alpha$ 1,2- and  $\alpha$ 1,3/4-  
306 linked-fucose residues in the HBGAs.



307 **Determination of BNeV-binding HBGA epitopes in bovine duodenal epithelium.**

308 The above results showed that the BNeV VLPs had no binding specificity for terminal SAs  
309 on the cultured cell surface. To confirm these results in bovine intestinal tissues, whether  
310 removal of terminal or internal parts of cell surface carbohydrate moieties using 1 or 10 mM  
311 NaIO<sub>4</sub> pretreatment could influence the attachment of BNeV to bovine duodenal epithelium  
312 was examined (23). Similar to the aforementioned findings (Fig. 2), abolishment of BNeV  
313 binding to the duodenal epithelium was achieved with the 10 mM but not the 1 mM NaIO<sub>4</sub>  
314 pretreatment (data not shown), suggesting that neutral sugars are also involved in binding to  
315 gut tissue. Consistent with this finding, removal of  $\alpha$ 2,3- and  $\alpha$ 2,6-linked SAs using NA from  
316 *V. cholera* failed to inhibit BNeV VLP binding to gut epithelium (data not shown).

317 We previously demonstrated that bovine duodenal epithelium expresses A type, H type  
318 2, Le<sup>y</sup>, and  $\alpha$ Gal HBGAs but no other HBGAs by immunohistochemistry (23). Confirming  
319 our previous results (23), bovine duodenal sections had two ABO blood types, H+/A-/B-  
320 /Le<sup>y</sup>+/ $\alpha$ -Gal+ and H+/A+/B-/Le<sup>y</sup>+/ $\alpha$ -Gal+ (data not shown). These results are consistent  
321 with the above findings for the HBGA phenotypes in the saliva samples, with A and O blood  
322 types being present in cattle (Fig. 6).

323 To determine whether BNeV VLPs can recognize HBGAs by binding to  $\alpha$ 1,2-linked-  
324 fucose,  $\alpha$ 1,3/4-linked-fucose,  $\alpha$ Gal, or GalNAc epitopes of HBGAs, bovine duodenal paraffin  
325 sections showing either O blood type (H+/A-/B-/Le<sup>y</sup>+/ $\alpha$ -Gal+) or A blood type (H+/A+/B-/  
326 Le<sup>y</sup>+/ $\alpha$ -Gal+) were selected and then pretreated with  $\alpha$ 1,2-L-fucosidase,  $\alpha$ 1,3/4-L-fucosidase,  
327  $\alpha$ -galactosidase, or  $\alpha$ N-acetylgalactosaminidase either individually or in dual combination.  
328 As found for the inhibitory effects of HBGA epitope-specific enzymes against BNeV binding  
329 to synthetic HBGAs (Fig. 4B), bovine and human saliva samples (Fig. 8), or cultured cells

330 (Fig. 9), pretreatment of O or A blood type duodenal sections with  $\alpha$ -galactosidase or  $\alpha$ N-  
331 acetylgalactosaminidase produced no inhibitory BNeV binding effects (Fig. 11). However,  
332 pretreatment of these duodenal sections with  $\alpha$ 1,2-L-fucosidase significantly reduced BNeV  
333 VLP binding. Pretreatment of duodenal sections with  $\alpha$ 1,3/4-L-fucosidase partially inhibited  
334 the binding of BNeV VLPs to the duodenal epithelium, whilst pretreatment of the duodenal  
335 sections with a combination of  $\alpha$ 1,2-L-fucosidase and  $\alpha$ 1,3/4-L-fucosidase almost completely  
336 abolished the binding of BNeV VLPs to the duodenal epithelium (Fig. 11). Taken together,  
337 these results indicate that BNeV VLPs can attach to bovine as well as human glycan  
338 compounds on the surface of epithelial cells containing  $\alpha$ 1,2-linked-fucose and  $\alpha$ 1,3/4-linked-  
339 fucose epitopes, as found in most HBGA epitopes.

## 340 **DISCUSSION**

341 Viruses must attach to cell surface attachment factor(s) and/or receptor(s) to initiate  
342 viral entry and replication (1, 2). Many caliciviruses attach to cell surface carbohydrate  
343 moieties, such as HBGAs or SAs (2, 28). Here, we demonstrate that VLPs of the BNeV bind  
344 to a wide spectrum of HBGAs present as synthetic neoglycoconjugates, secreted in bovine  
345 and human saliva, or expressed by cultured cells and bovine duodenal epithelium. Among  
346 HBGA-dependent caliciviruses, some HuNoVs, particularly some GII.4 strains and the  
347 recently emerged GII.17 strains, are well known to have a wide binding spectrum of HBGAs  
348 in all ABO, Lewis, and secretor/nonsecretor types (2, 58, 61, 62). Generally, it is considered  
349 that the multiple binding patterns of HuNoVs to HBGAs could be subdivided into two major  
350 binding groups: the A/B or Lewis binding groups (2, 51, 52). The A/B binding group  
351 members, including VA387, Norwalk, and MOH strains, are considered to accommodate one  
352 or two epitopes of A/B and H HBGAs, i.e., galactose and/or  $\alpha$ 1,2-linked-fucose. In contrast,  
353 the Lewis binding group members, such as the Boxer, VA207, and OIF strains, utilize  $\alpha$ 1,2-  
354 linked-fucose and/or  $\alpha$ 1,3-linked-fucose epitopes of H and Lewis HBGAs. In the present  
355 study, enzymatic removal of  $\alpha$ 1,2- and  $\alpha$ 1,3/4-linked-fucoses from the various HBGA  
356 backbones reduced BNeV binding to the corresponding synthetic HBGAs and saliva samples.  
357 These data indicate that similar to the human Lewis binding group, BNeV VLPs recognize a  
358 wide spectrum of HBGAs via binding to their  $\alpha$ 1,2- and  $\alpha$ 1,3/4-linked-fucose epitopes (2, 51,  
359 52, 58, 61). As expected, removal of the  $\alpha$ Gal or GalNAc epitopes had no inhibitory effect on  
360 BNeV VLP binding, indicating that BNeV does not use the  $\alpha$ Gal and GalNAc epitopes as  
361 attachment factors. Indeed, it is well known that bovine genogroup III NoV uses the  $\alpha$ Gal

362 epitope, which is absent from human and pig gut epithelium, suggesting that neither man nor  
363 pig could be infected by bovine NoVs (23). However, this remains to be explored.

364 The binding of BNeV VLPs to the bovine duodenal sections was markedly decreased  
365 by pretreatment with  $\alpha$ 1,2-L-fucosidase. This was attributed to the A type/H type 2/Le<sup>y</sup>  
366 HBGAs from the four types being expressed in bovine duodenal epithelium containing the  
367  $\alpha$ 1,2-linked-fucose epitope. In addition, there is a slight reduction in BNeV binding to the  
368 bovine duodenal sections with the  $\alpha$ 1,3/4-L-fucosidase pretreatment because the  $\alpha$ 1,3/4-  
369 linked-fucose epitope is present only in Le<sup>y</sup> HBGA. However, pretreatment of bovine  
370 duodenal sections with  $\alpha$ -galactosidase or  $\alpha$ N-acetylgalactosaminidase had no inhibitory  
371 effect on the binding of BNeV VLPs to the duodenal sections, indicating that the  $\alpha$ Gal  
372 epitope in Gal $\alpha$ 3Gal $\beta$ 4GlcNAc $\beta$  HBGA and the GalNAc epitope in A type HBGA were not  
373 be used as attachment epitopes for BNeV binding to the bovine duodenum. These findings  
374 also support the conclusion that similar to the human Lewis binding group, BNeV VLPs  
375 attach to  $\alpha$ 1,2- and/or  $\alpha$ 1,3/4-linked-fucose epitopes in H type 2/Le<sup>y</sup> HBGAs expressed in the  
376 bovine duodenal epithelium (2, 51, 52, 58, 61).

377 As demonstrated in the present study, BNeV had a wide HBGA binding spectrum  
378 through recognition of  $\alpha$ 1,2-linked-fucose and  $\alpha$ 1,3/4-linked-fucose epitopes of targeted  
379 HBGAs. It should be noted that bovine and human saliva samples have different levels of  
380 ABH and Lewis antigens which carry  $\alpha$ 1,2-linked-fucose and/or  $\alpha$ 1,3/4-linked-fucose  
381 epitopes (23). Therefore, pretreatment of bovine and human saliva samples with a mixture of  
382  $\alpha$ 1,2-L-fucosidase and  $\alpha$ 1,3/4-L-fucosidase should have higher inhibitory effects on BNeV  
383 binding than that with either  $\alpha$ 1,2-L-fucosidase or  $\alpha$ 1,3/4-L-fucosidase individually.

384           Although the synthetic HBGA binding assay showed that the VLPs from BNeV had  
385 strong binding to SLe<sup>a</sup> HBGA and weak binding to SLe<sup>x</sup> HBGA, they were expressed in the  
386 bovine small intestinal epithelium or secreted into saliva with the methods used. This means  
387 that BNeV does not use SLe<sup>a</sup> and SLe<sup>x</sup> for entry and infection in cattle. Both SLe<sup>a</sup> and SLe<sup>x</sup>  
388 are expressed at high concentrations in cancer cells such as human colon cancer but only  
389 minimally in non-transformed cells, defining their utility as diagnostic cancer markers in  
390 human medicine (63). The reason for the binding of BNeV VLPs to SLe<sup>a</sup> and SLe<sup>x</sup> is the  
391 presence of  $\alpha$ 1,3/4-linked-fucose epitopes (57).

392           SA-containing gangliosides have been identified as attachment factors for murine  
393 MNV1, PSaV, and FCV (26–28). Interestingly, recent studies have shown that in addition to  
394 HBGAs, SAs can be also used as attachment factor(s) for HuNoVs and Tulane virus, the  
395 prototype of the *Recovirus* genus;  $\alpha$ 2,3- and  $\alpha$ 2,6-linked SAs, particularly  $\alpha$ 2,3-linked SA  
396 containing GM3, possibly act as attachment factors for HuNoV VA387 (GII.4) and VA115  
397 (GI.3) strains, whereas  $\alpha$ 2,6-linked terminal SAs are likely utilized by Tulane virus (29, 30).  
398 In contrast to the reduced binding observed for the Tulane virus (29), in this study, removal  
399 of terminal SAs from cell surface carbohydrates by NA and 1 mM NaIO<sub>4</sub> had no inhibitory  
400 effect on the binding of BNeV VLPs to the cells. Rather, pretreatment with NA and 1 mM  
401 NaIO<sub>4</sub> increased BNeV VLP binding. This may be due to increased access to fucosylated  
402 epitopes following removal of the charged SA motifs. However, pretreatment with 10 mM  
403 NaIO<sub>4</sub> markedly decreased the BNeV binding to the cells, possibly due to complete removal  
404 of HBGAs on the cell surface (23). The lack of inhibition following NA treatment does not  
405 rule out the possibility that the internal SAs of gangliosides like GM1a are recognized (64).

406 However, the lack of sensitivity with the 1 mM NaIO<sub>4</sub> treatment strongly suggests that this is  
407 not the case.

408 In the present study, we demonstrate that BNeV VLPs do not recognize  $\alpha$ Gal epitope.  
409 Additionally, MDBK cells were found to solely express  $\alpha$ Gal epitope, and not to express  
410 fucosylated HBGA with the antibodies used. Therefore, it was anticipated that  $\alpha$ 1,2- and  
411  $\alpha$ 1,3/4-linked fucose epitope-dependent BNeV VLPs would not bind the cell surface of  
412 MDBK cells. Unexpectedly, however, BNeV VLPs attached to MDBK cells and pretreatment  
413 of MDBK cells with these fucosidases inhibited BNeV binding to MDBK cells. The  
414 mechanism by which BNeV VLPs bind to MDBK cells remains unclear. Nevertheless, these  
415 data suggest that MDBK cells express fucosylated HBGAs, which could not be detected by  
416 the antibodies used in this study but could be degraded by fucosidases. The identification of  
417 specific fucose-containing epitopes on MDBK cells involved in BNeV binding forms the  
418 basis of an ongoing work.

419 Because infection by all viruses begins with the attachment of the virus to the host cells,  
420 expression levels of the target receptor for a given virus could be an important factor in  
421 determining the viral tropisms, pathogenesis, and host range restriction (6, 66–68). Some  
422 NoVs detected in humans and animals have a close genetic relationship and share target  
423 receptors, which has raised the questions regarding the possibility of the zoonotic  
424 transmission of these viruses (24, 69). For example, porcine NoV strains detected worldwide  
425 have close genetic relatedness to HuNoVs (70–77). Moreover, HuNoV strain GII.4-HS66  
426 induces diarrhea and intestinal pathology in piglets and calves, respectively (78, 79). Recent  
427 studies have shown that primate enteric caliciviruses within the *Recovirus* genus share their  
428 HBGA attachment factors with HuNoVs (25, 80). Furthermore, the zoonotic potential of

429 these viruses has been clearly demonstrated by the high prevalence of neutralizing antibodies  
430 (81, 82). In the present study, BNeV utilized HBGAs that are commonly used by HuNoVs  
431 and other enteric caliciviruses. This suggests that BNeVs have the potential to infect humans  
432 and/or other species. In particular, VLPs from BNeV displayed wide-spectrum binding in  
433 human saliva, and human and animal cell lines, further supporting the above hypothesis. It  
434 should be noted that bovines express type 2-based HBGAs in their digestive tract but humans  
435 have type 1-based structures in their digestive tract. Accordingly, HuNoVs appear to favor  
436 the recognition of type 1-based HBGAs (83, 84). BNeVs might be less adapted to human  
437 infection, as neither the virus nor its specific antibody have been isolated from and detected  
438 in humans.

439 To demonstrate the direct interspecies transmission of BNeVs, the molecular detection  
440 of BNeVs and sequence analysis of resultant amplicons are necessary in stool samples of  
441 humans and animals such as pigs, particularly where humans and animals or different animal  
442 species live in close physical contact and mixed infections are more common (82). Although  
443 robust and reproducible in vitro cultivation systems for BNeVs have not been established,  
444 more definitive evidence for the interspecies transmission of BNeVs can be obtained by the  
445 inoculation of the BNeV isolates into different species such as human volunteers or piglets.  
446 Indirect evidence for interspecies transmission of BNeVs can be provided by the detection of  
447 antibodies against BNeVs in serum samples collected from humans and other animals,  
448 particularly those living in the above environments (81, 85, 86). Nevertheless, our results  
449 stress the need for more in-depth genomic and serological studies of BNeVs in humans and  
450 other species.

451 In conclusion, the present study provides direct evidence that BNeV VLPs attach to H  
452 type 2/Le<sup>y</sup>/Le<sup>x</sup> HBGAs expressed in the bovine digestive tract through their  $\alpha$ 1,2- and  $\alpha$ 1,3/4-  
453 linked fucose residues. Moreover, the usage of multiple HBGAs by BNeV VLPs and their  
454 ability to bind to human saliva suggest that BNeVs may have the potential for zoonotic  
455 transmission. More in-depth epidemiological studies using human fecal and serum samples  
456 are required to determine the zoonotic potential of BNeVs. Similarly, continued  
457 investigations regarding the proteinaceous receptor(s) are necessary for a better  
458 understanding of the tropism, pathogenesis, and host range of this important viral genus.



459 **MATERIALS AND METHODS**

460 **Cells and viruses.** Madin-Darby bovine kidney (MDBK), porcine kidney LLC-PK, and  
461 human cervical cancer HeLa cells purchased from American Type Culture Collection (ATCC,  
462 USA) were maintained in Eagle's minimum essential medium supplemented with 10% fetal  
463 bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin as described  
464 elsewhere (7, 87). Madin-Darby canine kidney (MDCK), Crandell-Reese feline kidney  
465 (CRFK), human embryonic kidney 293T (HEK293T), and human colorectal adenocarcinoma  
466 Caco-2 cells purchased from ATCC were grown in Dulbecco's modified Eagle's medium  
467 (DMEM) supplemented with 5% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (7,  
468 86). The parental Chinese hamster ovary (CHO) cells that do not express HBGAs (H-/A-/B-)  
469 were maintained in RPMI1640 supplemented with 10% FBS, 1% L-glutamine, 100 U/ml  
470 penicillin, 100 µg/ml streptomycin, and 10 µg/ml each of adenosine, 2-deoxy-adenosine and  
471 thymidine (60). In addition, single-transfectant CHO cells expressing the H antigen (H+/A-  
472 /B-) or the αGal antigen, and double-transfectant CHO cells expressing either the A antigen  
473 (H+/A+/B-) or the B antigen (H+/A-/B+) were cultured in the conditions described for the  
474 parental CHO cells, with addition of 0.2 mg/ml hygromycin and 0.25 mg/ml of G418  
475 (neomycin) to maintain the plasmids expressing the glycosylation enzymes (60). Sf9 cells,  
476 purchased from Gibco (Fort Worth, Texas, USA), were cultured at 27°C in SF-900 II SFM  
477 media containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, lipid medium  
478 supplement, and 0.1% pluronic acid solution (Sigma Aldrich, St. Louis, MO, USA). The FCV  
479 F9 strain (ATCC) and CVB3 Nancy strain were propagated in CRFK cells and HeLa cells,  
480 respectively (26, 88). Cesium chloride (CsCl) density gradient ultracentrifugation was used to  
481 purify each mass-cultured strain as described elsewhere (7).

482           **Reagents and antibodies.** NaIO<sub>4</sub> and NA from Sigma-Aldrich were dissolved in PBS  
483 (pH 7.2). Alpha1,2-L-fucosidase from *Corynebacterium* (Takara Bio Inc., Kyoto, Japan),  
484  $\alpha$ 1,3/4-L-fucosidase from *Streptomyces* (Takara Bio Inc.),  $\alpha$ -galactosidase from *Coffea*  
485 *arabica* (Sigma-Aldrich),  $\alpha$ N-acetylgalactosaminidase from *Chryseobacterium*  
486 *meningosepticum* (New England BioLabs, Inc., MA, USA), and UEA-1 (Vector Laboratories,  
487 Burlingame, CA, USA) were diluted in PBS. AF594 succinimidyl ester, purchased from  
488 Molecular Probes (Eugene, OR, USA), was dissolved in DMSO. [<sup>35</sup>S] methionine/cysteine  
489 was purchased from PerkinElmer (Waltham, MA, USA). Biotin-conjugated oligosaccharides  
490 including Lewis antigens (Le<sup>a</sup>, Le<sup>b</sup>, Le<sup>x</sup>, and Le<sup>y</sup>), H type, type A disaccharide, type B  
491 disaccharide, type A trisaccharide, type B trisaccharide,  $\alpha$ Gal trisaccharide, sialyl-Le<sup>a</sup> (SL<sup>a</sup>),  
492 and SLe<sup>x</sup> tetrasaccharides were purchased from GlycoTech (Gaithersburg, MD, USA) (7).  
493 The following antibodies were used in this study: hyperimmune sera against BNeV capsid  
494 protein, P particles of human NoV VA387 strain, and VP8\* protein of human rotavirus Wa  
495 strain generated from rabbits by serial inoculation with each target protein as described  
496 below, monoclonal antibodies (Mabs) of anti-GST (Santa Cruz Biotechnology), anti-blood  
497 group A type antigen (types 1 and 2 chains) (Covance, NJ, USA), anti-blood group H antigen  
498 (type 1 chain) (Covance), anti-Le<sup>a</sup> antigen (type 1 chain) (Covance), anti-Le<sup>b</sup> antigen (type 1  
499 chain) (Covance), anti-Le<sup>x</sup> antigen (type 2 chain) (Covance), anti-Le<sup>y</sup> antigen (type 2 chain)  
500 (Covance), anti-blood group B antigen (Thermo Scientific, MA, USA), anti-H type 2 antigen  
501 (Thermo Scientific), anti- $\alpha$ Gal epitope antigen (Enzo Life Sciences, Seoul, South Korea),  
502 anti-GAPDH Mab (Santa Cruz Biotechnology), anti-rabbit IgG-fluorescence isothiocyanate  
503 (FITC)-conjugated antibody (Jackson Immunoresearch Lab, West Grove, PA, USA),  
504 biotinylated goat anti-mouse or anti-rabbit antibodies (Dako, Glostrup, Denmark), and anti-  
505 mouse IgG-FITC-conjugated antibody (Santa Cruz Biotechnology). Horseradish peroxidase

506 (HRP)-conjugated streptavidin and HRP-conjugated goat anti-rabbit immunoglobulin G (IgG)  
507 and anti-mouse IgG antibodies were obtained from the Jackson Immunoresearch Lab or Dako.

508 **Treatment of cells with chemicals and enzymes.** To determine whether BNeVs  
509 recognize terminal SAs as attachment factors, the following methods were used as described  
510 previously (7). Cells were treated with 1 or 10 mM NaIO<sub>4</sub> for 30 min at 4°C or with NA at  
511 100 mU for 1 hr at 37°C in PBS. After the pretreatment, cells were washed three times with  
512 PBS. The binding assays were then carried out as described below. Mock and control  
513 treatments were performed at the same time.

514 **Expression and purification of BNeV VLPs.** BNeV VLPs were generated from a calf  
515 diarrhea fecal sample determined positive for BNeV by a PCR based method (38) as  
516 described elsewhere (48, 49). Briefly, the complete 2.3 kb capsid region (encoding the VP1-  
517 major capsid and VP2-minor capsid regions) of Bo/BNeV/MA415/04/KR was amplified  
518 from the above fecal sample by reverse transcription polymerase chain reaction (RT-PCR)  
519 with forward (5'-AAACATGAGTGACAACAAAAGCATCCCAGA-3', nucleotide position  
520 5055 to 5084 of VP1 region) and reverse (5'-  
521 TCAAACACTCGTGGTTCGAGAACACTGAC-3', nucleotide position 7360 to 7387 of VP2  
522 region) primers designed from the full-length sequence of Newbury agent 1 strain in the  
523 Genbank database (accession number NC\_007916). The amplicon was ligated into the  
524 pCR2.1-TOPO vector (Invitrogen, CA) and then transformed to DH5 $\alpha$  competent cells  
525 (Enzymomics, Daejeon, Korea). Plasmids were purified using GeneAll Hybrid-Q Plasmid  
526 Rapidpre (GeneAll, Seoul, Korea) and the sequence (GenBank accession numbers EF528565,  
527 MG009451) was verified using an ABAI system 3700 automated DNA sequencer (Applied  
528 Biosystems, Foster City, USA). Using purified plasmid, the full length cDNA copy of capsid

529 gene was amplified by PCR with forward primer (5'-  
530 CACAGGATCCATGAGTGACAACAAAAGCAT-3') containing BamHI restriction site  
531 (underlined) and reverse primer (5'-AATCTCGAGTCAAACACTCGTGGTCG-3')  
532 containing XhoI restriction site (underlined). After digestion with BamHI and XhoI  
533 restriction enzymes, the amplified fragments were subcloned into pFastBac1 baculovirus  
534 donor plasmid (ThermoFisher Scientific, Seoul, South Korea). The pFastBac1 donor plasmid  
535 was transformed into DH10Bac *Escherichia coli* and its resultant recombinant bacmid DNA  
536 was transfected into Sf9 cells using Cellfectin II reagent (Invitrogen; following  
537 manufacturer's instruction). BNeV VLPs were expressed in baculovirus recombinant-  
538 transformed Sf9 insect cells at 27°C and harvested at 5-7 days post-infection. The cloned  
539 recombinant baculovirus generated from pFastBac1 plasmid containing VP1 and VP2 regions  
540 of MA415 strain was designed as rMA415 and propagated in Sf9 cells to make master virus  
541 stocks. BNeV VLPs were purified using CsCl density gradient ultracentrifugation as  
542 described elsewhere (7). The protein concentrations of the VLPs were determined with a  
543 BCA protein assay kit (Pierce, IL, USA) according to the manufacturer's instruction.  
544 Expression of recombinant capsid protein was validated by electron microscopy,  
545 immunofluorescence, and Western blot analyses as described elsewhere (48, 49, 89).

546 **Electron microscopy.** VLPs purified from rMA415-infected Sf9 cell culture  
547 supernatants by CsCl density gradient ultracentrifugation were stained with 3%  
548 phosphotungstic acid (pH 7.0) and examined with an electron microscope (JEM-2000 FXII,  
549 JEOL, USA) as described previously (49).

550 **Production of rabbit hyperimmune antiserum.** A rabbit hyperimmune antiserum  
551 against BNeV VLPs, P particles of human NoV VA387 strain, and VP8\* protein of human

552 rotavirus Wa strain was performed as described elsewhere (48, 49). Briefly, two rabbits for  
553 each target viral protein were subcutaneously immunized three times with purified BNeV  
554 VLPs, P particles of HuNoV, or VP8\* protein of rotavirus in complete Freund's adjuvant for  
555 the first injection or incomplete Freund's adjuvant for the subsequent infections. The animals  
556 were bled 2 weeks after the last booster injection.

557 **Coomassie blue staining and Western blot analysis.** To check the quality and expression of  
558 BNeV VLPs, Coomassie blue staining and Western blot analysis were performed as  
559 described elsewhere (48, 49, 89). Briefly, the proteins in the supernatant of cells infected with  
560 rMA415 or wild-type baculovirus were concentrated by precipitation with 8% polyethylene  
561 glycol. The cells infected with rMA415 or wild-type baculovirus were washed three times  
562 with cold PBS and lysed using cell extraction buffer containing 10 mM Tris/HCl pH 7.4, 100  
563 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1%  
564 Triton X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate (Invitrogen) for 30 min on  
565 ice. Lysates were spun down by centrifugation at 12,000×g for 10 min at 4°C and the samples  
566 were analyzed for total protein content with a BCA protein assay kit (Thermo Scientific,  
567 Waltham, MA, USA). Samples were resolved by SDS-PAGE and served for Coomassie blue  
568 staining or transferred onto nitrocellulose membranes (GE Healthcare Life Sciences). The  
569 membranes were blocked for 1 hr at room temperature with Tris-buffered saline containing 5%  
570 skimmed milk before they were incubated overnight at 4°C with the primary rabbit  
571 polyclonal antibody against BNeV capsid protein. The bound antibody was developed by  
572 incubation with a HRP-labeled secondary antibody, and the immunoreactive bands were  
573 detected by enhanced chemiluminescence (ECL) (Dogen, Seoul, South Korea) using a  
574 Davinch-K Imaging System (Youngwha Scientific Co., Ltd, Seoul, South Korea).

575           **Expression and purification of the GST-P particle and GST-VP8\* protein.** The  
576 GST-P particles of the HuNoV strain VA387 (GII.4) and the GST-VP8\* proteins of the  
577 human rotavirus P[25] Dhaka6 and bovine rotavirus P[5] WC3 strains were cloned, expressed,  
578 and purified as described previously (55, 90). The concentration of the purified NoV P  
579 particles and VP8\* proteins of the rotavirus strains were determined using a BCA protein  
580 assay kit (Pierce, IL, USA) according to the manufacturer's instruction.

581           **AF594 labeling of viruses and VLPs.** The FCV F9 and CVB3 Nancy strains, and  
582 BNeV VLPs purified by CsCl density gradient ultracentrifugation were labeled with AF594  
583 as described previously (7). Briefly, purified virus particles and VLPs (10 mg at 1 mg ml<sup>-1</sup>) in  
584 0.1 M sodium bicarbonate buffer (pH 8.3) were labeled with a one-tenth fold-molar  
585 concentration of AF594 succinimidyl ester (1 mg at 1 mg ml<sup>-1</sup> in DMSO). After thorough  
586 vortexing for 30 sec, each mixture was incubated for 1 hr at room temperature with  
587 continuous stirring. Labeled viruses and VLPs were repurified by CsCl density gradient  
588 ultracentrifugation, dialyzed, and stored in 2 µg aliquots at -20°C (91). The concentration of  
589 the purified AF594-labeled BNeV VLPs, FCV strain, and CVB3 strain was determined using  
590 a BCA protein assay kit (Pierce, IL, USA) according to the manufacturer's instructions.  
591 Analysis of SDS-PAGE-separated, AF594-labeled viral particles and VLPs using Coomassie  
592 blue staining and Western blotting showed that the label was exclusively coupled to each  
593 viral protein.

594           **Immunofluorescence assay.** To determine the expression levels of each HBGA antigen  
595 on the cell surface, the binding specificity of AF594-labeled BNeV VLPs, FCV strain, and  
596 CVB3 strain to various cell lines including transfectant CHO cells, and the expression levels  
597 of BNeV VLPs in the Sf9 cells infected with rMA415, the immunofluorescence assay was

598 performed as described elsewhere (87, 89). Briefly, the confluent cells grown on eight-  
599 chamber slides were treated with or without chemicals or enzymes and then fixed with 4%  
600 paraformaldehyde in PBS for 1 hr. For detection of the BNeV capsid proteins in the Sf9 cells,  
601 mock- or rMA415-infected Sf9 cells grown on microscope cover slides were harvested at 48  
602 and 72 hr post-infection and then fixed with 4% paraformaldehyde in PBS for 1 hr. The cells  
603 were then permeabilized by the addition of 0.2% Triton X-100 and washed with PBS  
604 containing 0.1% newborn calf serum (PBS-NCS). Mabs specific for each HBGA and a  
605 polyclonal antibody against BNeV capsid protein were added to each chamber or cover slides  
606 and the slides were incubated at 4°C overnight. The cells were then washed three times with  
607 PBS-NCS and FITC-conjugated secondary antibodies were added. After washing, the cells  
608 were treated with DAPI solution for the staining of nuclei, mounted using SlowFade Gold  
609 antifade reagent (Invitrogen), and then examined under an EZ-C1 confocal microscope using  
610 EZ-C1 software (Nikon, Japan). Another set of eight chamber slides treated described above  
611 was added with AF594-labeled BNeV VLPs and were used to observe binding as described  
612 above. For detecting the BNeV capsid protein in the infected Sf9 cells, mock- or rMA415-  
613 infected Sf9 cells were incubated with FITC-conjugated secondary antibody. After washing,  
614 the cells were mounted with 60% glycerol in PBS (pH 8.0), and then examined under a  
615 fluorescence microscope.

616       **Labeling of viruses and VLPs with [<sup>35</sup>S] methionine/cysteine.** Radioisotope labeling  
617 of the FCV F9 and CVB3 Nancy strains with [<sup>35</sup>S] methionine/cysteine (PerkinElmer) was  
618 carried out as described elsewhere (7, 26). Briefly, each individual virus was inoculated at an  
619 MOI of 0.1 PFU/cell into confluent monolayers of cells and incubated for 4 hr at 37°C. The  
620 medium was replaced with RPMI 1640 lacking methionine and cysteine (Sigma-Aldrich).

621 Cells were starved for 2 hr and then supplemented with 1 Mbq [<sup>35</sup>S]methionine/cysteine ml<sup>-1</sup>  
622 (PerkinElmer). At 72 hr post-infection, each labeled virus was purified by CsCl density  
623 gradient ultracentrifugation as described (7). BNeV VLPs metabolically radiolabeled with  
624 [<sup>35</sup>S] methionine/cysteine (PerkinElmer) were prepared as described previously with slight  
625 modifications (92). Briefly, Sf9 cells were infected with recombinant baculovirus at an MOI  
626 of 10 PFU per cell and then incubated for 28 hr. The medium was replaced with non-  
627 supplemented Grace's insect medium (Gibco). Cells were starved for 30 min and then 30 µCi  
628 [<sup>35</sup>S]methionine/cysteine ml<sup>-1</sup> (PerkinElmer) were added. At 4 to 6 hr following radioisotope  
629 labeling, the medium was volumetrically replaced with the same amount of Grace's insect  
630 medium with Sf-900 II SFM (Gibco). The cultures were harvested when 80% of the cells  
631 showed cytopathic effects. Radioisotope-labelled VLPs were purified by CsCl density  
632 gradient ultracentrifugation as described (7). The concentration of the purified labeled BNeV  
633 VLPs, FCV strain and CVB3 strain was determined using a BCA protein assay kit (Pierce)  
634 according to the manufacturer's instruction.

635 **Attachment assay with [<sup>35</sup>S]methionine/cysteine-labeled VLPs and viruses.** Binding  
636 of <sup>35</sup>[S]methionine/cysteine-labeled BNeV VLPs, and the FCV F9 and CVB3 Nancy strains  
637 to each corresponding cell line was performed as described elsewhere (7). Briefly, cells (4 x  
638 10<sup>4</sup>/well) were plated into 96-well microtiter plates and then independently incubated with  
639 purified [<sup>35</sup>S]methionine/cysteine-labeled BNeV VLPs, and the FCV and CVB3 strains (50  
640 000 c.p.m.) for 45 min on ice. Cells were washed three times with ice-cold PBS followed by  
641 cell lysis with 0.1% sodium dodecyl sulfate and 0.1 M NaOH. Total radioactivity in the cell  
642 lysate was determined by liquid scintillation counting.

643 **Synthetic HBGA binding assay.** To determine the binding specificity of BNeV VLPs,



644 the GST-VP8\* proteins of the human rotavirus P[25] strain Dhaka6 and the GST- P particles  
645 of the HuNoV strain VA387 to each HBGA, a synthetic oligosaccharide-based HBGA  
646 binding assay was carried out as described elsewhere (7). Briefly, 96-well microtiter plates  
647 were coated with 50 µg/ml BNeV VLPs, 10 µg/ml of each GST-tagged-VP8\* protein and  
648 GST-tagged P particles, the supernatant of wild-type baculovirus-infected Sf9 cell lysate, and  
649 GST and then incubated at 4°C overnight. Coated plates were blocked with 5% bovine serum  
650 albumin (BSA) for 1 hr at room temperature, and each synthetic oligosaccharide-  
651 polyacrylamide (PAA)-biotin conjugate (10 µg/ml) was then added and further incubated at  
652 4°C overnight. Bound oligosaccharides were detected using HRP-conjugated streptavidin.  
653 The signal intensities were visualized by 3,3',5,5'-tetramethylbenzidine (TMB,  
654 Komabiotech), and the absorbance was read at 450 nm wavelength in a plate reader. For each  
655 step, the plates were incubated at 37°C for 1 hr and washed five times with PBS containing  
656 0.05% Tween 20 (PBS-Tween 20).

657 **Determination of binding epitopes in each synthetic HBGA.** To determine the target  
658 HBGA epitopes for the BNeV VLPs, removal of each epitope from the synthetic HBGAs was  
659 performed as described previously with slight modifications (23, 93, 94). Briefly, 96-well  
660 plates were coated with each of the synthetic HBGAs and incubated at 4°C for 6 hr. The  
661 plates were washed thrice with PBS-Tween 20 and blocked with PBS-BSA. After washing  
662 three times with PBS, the coated plates were incubated with 100 µl solution containing 20  
663 mU/ml of  $\alpha$ -1,2-L-fucosidase, 10 µU/ml  $\alpha$ -1,3/4-L-fucosidase, 3 mU/ml  $\alpha$ -galactosidase or 5  
664 mU/ml  $\alpha$ -N-acetylgalactosaminidase for 24 hr at 37°C. Thereafter, the plates were washed  
665 thrice with PBS and incubated with 50 µg/ml of BNeV VLPs at 4°C for 1 hr. After washing  
666 with PBS, the plates were incubated with a hyperimmune serum against BNeV capsid protein.

667 Following this, the plates were washed thrice with PBS and treated with HRP-conjugated  
668 goat anti-rabbit IgG. The signals were visualized by TMB followed by absorbance  
669 measurement at 450 nm wavelength using a plate reader.

670 **Saliva binding assay.** Saliva samples from 53 human individuals and 8 cows were  
671 selected from the Archives of the Saliva Registry of the Laboratory of Veterinary Pathology,  
672 College of Veterinary Medicine, Chonnam National University, Gwangju. Before performing  
673 the saliva binding assay, the amount of each HBGA content in saliva samples was determined  
674 by enzyme immunoassays as described previously (51, 52, 56). Briefly, boiled saliva samples  
675 were diluted to 1:20 in PBS and then coated onto microtiter immuno plates (Thermo Fisher  
676 Scientific) at 4°C overnight. After blocking with PBS containing 5% BSA at 37°C for 1 hr,  
677 Mabs specific to H1, H2, Le<sup>a</sup>, Le<sup>b</sup>, Le<sup>x</sup>, Le<sup>y</sup>, type A, type B, and  $\alpha$ -Gal HBGAs were added to  
678 each well and the plates were incubated for 1 hr at 37°C. After washing, HRP-conjugated  
679 goat anti-mouse anti-IgG or IgM were added to each well. After each step, the plates were  
680 washed five times with PBS. The color reaction after substrate addition was measured as  
681 described above.

682 Binding of the BNeV VLPs, GST-P particles of the HuNoV strain VA387, GST-VP8\*  
683 proteins of the human rotavirus P[25] strain Dhaka6, and GST-VP8\* proteins of the bovine  
684 rotavirus P[5] strain WC3 was assessed using the saliva binding assay as described  
685 previously with slight modification (51, 52, 56). Briefly, boiled saliva samples were diluted to  
686 1:20 and then coated onto 96-well plates at 4°C overnight. After blocking with PBS-BSA at  
687 37°C for 1 hr, 50  $\mu$ g/ml of the BNeV VLPs and 10  $\mu$ g/ml of each viral protein were added,  
688 followed by incubation for 1 hr at 37°C. The bound target proteins were detected using an  
689 anti-GST antibody or hyperimmune serum against BNeV capsid protein diluted to 1:1000,

690 followed by addition of HRP-conjugated goat anti-mouse or anti-rabbit IgG antibodies. The  
691 signal intensities after addition of substrate were displayed by a TMB kit as described above.

692 **Determination of HBGA binding epitopes in each saliva samples.** To determine the  
693 target HBGA epitope for BNeV VLPs, removal of epitopes from selected bovine and human  
694 saliva samples was performed as described previously with slight modification (23, 24, 94,  
695 95). Briefly, boiled saliva samples were diluted to 1:20 and then coated onto 96-well plates at  
696 4°C overnight. The plates were washed thrice with PBS-Tween 20 and then incubated with  
697 100 µl solution containing 10 mU/ml of α1,2-L-fucosidase, 10 µU/ml of α1,3/4-L-fucosidase,  
698 4 mU/ml of α-galactosidase, or 8 mU/ml of α-N-acetylgalactosaminidase for 48 hr at 37°C.  
699 After blocking with PBS-BSA at 37°C for 1 hr, 50 µg/ml BNeV VLPs were added and  
700 incubated for 1 hr at 37°C. The bound target proteins were detected using a rabbit  
701 hyperimmune serum against BNeV capsid protein diluted to 1:1000, followed by addition of  
702 HRP-conjugated goat anti-rabbit IgG antibody. The signal intensities after substrate addition  
703 were obtained using a TMB kit as described above.

704 **Tissue samples and immunohistochemical analysis.** Paraffin-embedded bovine small  
705 intestinal samples obtained by necropsy from healthy calves were selected from the Archives  
706 of the Tissue Registry of the Laboratory of Veterinary Pathology, College of Veterinary  
707 Medicine, Chonnam National University, Gwangju. To determine the binding and inhibitory  
708 effects of NaIO<sub>4</sub> and the enzymes on the binding of BNeV VLPs to bovine small intestinal  
709 villous epithelial cells, immunohistochemical analysis was performed as described elsewhere  
710 (23, 28). Briefly, tissue sections of 3 µm thickness were deparaffinized, rehydrated through a  
711 graded ethanol series, and washed in PBS. Thereafter, the sections were treated with 0.3%  
712 H<sub>2</sub>O<sub>2</sub> in methanol for 20 min to quench endogenous peroxidase, washed three times with PBS,

713 and blocked with PBS-BSA for 30 min at room temperature in a humid atmosphere to inhibit  
714 non-specific binding. To determine the expression of HBGAs, duodenal sections were first  
715 treated with the primary Mabs specific for each HBGA and left at 4°C overnight. Washed  
716 sections were also treated with or without either NaIO<sub>4</sub> at 1 mM or 10 mM in 50 mM sodium  
717 acetate buffer (pH 5.0) for 30 min at room temperature, followed by a 10 min incubation with  
718 1% glycine in PBS to remove terminal or internal cell surface carbohydrate moieties, or with  
719 enzymes for 18 hr at 37°C for removal of each HBGA epitope or cleaving terminal SAs from  
720 epithelial cells. After washing with PBS, the sections were incubated with 10 µg/ml BNeV  
721 VLPs, diluted in PBS-BSA, and left at 4°C overnight. They were then washed thrice with  
722 PBS-BSA and incubated with a rabbit hyperimmune serum against BNeV capsid protein at  
723 4°C overnight. After washing with PBS, the sections were incubated with biotinylated goat  
724 anti-rabbit or anti-mouse secondary antibodies (Dako) followed by peroxidase-conjugated  
725 streptavidin (Dako). The reactions were developed with 3,3'-diaminobenzidine  
726 tetrahydrochloride (DAB; Vector Laboratories) followed by treatment with Mayer's  
727 hematoxylin solution (Merck, Germany) for counterstaining.

728 **Ethics statement.** All animals were handled in strict accordance with good animal  
729 practices, as described in the NIH Guide for the Care and Use of Laboratory Animals (NIH  
730 Publication No. 85-23, 1985, revised 1996). The protocol was approved by the Committee on  
731 Ethics of Animal Experiments, CNU (permit number, CNU IACUC-YB-2016-65). The  
732 human saliva samples collected with written consent from volunteers were handled in strict  
733 accordance with human subjects, as described in the Guidance for the Care and Use of  
734 Human Samples of the CNU adhered from the WMA Declaration of Helsinki (Ethical  
735 Principles for Medical Research Involving Human Subjects). The protocol was approved by

736 the Committee for Research Ethics Concerning Human Subjects, CNU with permit number  
737 (CNU IBR No. 1040198-130807-BR-002-02).

738 **Statistical analyses and software.** Statistical analyses were performed on triplicate  
739 experiments using the GraphPad Prism software version 5.03 (GraphPad Software Inc., La  
740 Jolla, CA, USA) and a One–Way ANOVA test. *P* values of less than 0.05 were considered to  
741 be statistically significant. Figures were generated using Adobe Photoshop CS3 and Prism 5  
742 version 5.03.

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## 1052 **Figure Legends**

1053 **FIG 1** Electron micrograph of BNeV VLPs from and detection of BNeV capsid protein in  
1054 rMA415-infected insect cells by immunofluorescence assay. (A) The rMA415 VLPs were  
1055 purified by CsCl gradients from the cell culture supernatants of rMA415-infected Sf9 cells  
1056 and visualized by negative staining with 3% phosphotungstic acid (pH 7.0). The right panel is  
1057 a magnification of the left panel. (B) Sf9 cells were mock-infected (left panel) or infected  
1058 with rMA415. After 48 (middle panel) and 72 hr post-infection (right panel), cells were  
1059 immunostained using the rabbit hyperimmune serum against BNeV VLP and FITC-conjugated  
1060 goat anti-rabbit IgG antibody. The scale bars for left and right panels correspond to 50  $\mu\text{m}$ .

1061 **FIG 2** Binding of BNeV VLPs to cell surface carbohydrate. MDBK, CRFK, Caco-2 and  
1062 HeLa cells were pretreated with 1 mM or 10 mM  $\text{NaIO}_4$  to remove the carbohydrate moieties.  
1063 (A) Cells were incubated with the AF594-labeled BNeV VLPs, P particles of human NoV  
1064 VA387 strain, FCV F9 strain, and CVB3 Nancy strain at 10  $\mu\text{g}/\text{ml}$  and then examined by  
1065 confocal microscopy. (B) The [ $^{35}\text{S}$ ]Methionine/Cysteine-labeled BNeV VLPs, control FCV  
1066 F9 strain, or CVB3 Nancy strain (50 000 c.p.m.) were bound to MDBK, CRFK, or HeLa cells  
1067 following pretreatment with or without  $\text{NaIO}_4$ . Binding was quantified by liquid scintillation  
1068 counting. All experiments were performed three independent times, and Figure A shows one  
1069 representative set of results. The scale bars in Figure A correspond to 10  $\mu\text{m}$ . Error bars  
1070 indicate SD from triplicate samples. \* $p < 0.05$ , \*\* $p < 0.005$ .

1071 **FIG 3** Lack of BNeV VLP binding to cell surface terminal sialic acids (SAs). MDBK, CRFK,  
1072 Caco-2, and HeLa cells were pretreated with 100 mU *V. cholerae* neuraminidase (NA)  $\text{ml}^{-1}$   
1073 to remove  $\alpha 2,3$ -,  $\alpha 2,6$ - and  $\alpha 2,8$ -linked SAs from the carbohydrate moieties. (A) The AF594-

1074 labeled BNeV VLPs, FCV F9 strain, P particles of human NoV VA387 strain, and CVB3  
1075 Nancy strain were added to the cells at 10 µg/ml, and then examined by confocal microscopy.  
1076 (B) The [<sup>35</sup>S]Methionine/Cysteine-labeled BNeV VLPs, control FCV F9 strain, or CVB3  
1077 Nancy strain (50 000 c.p.m.) were bound to MDBK, CRFK, Caco-,2 or HeLa cells after  
1078 being pretreated with or without NA. Binding was measured by liquid scintillation counting.  
1079 All experiments were performed three independent times, and Figure A shows one  
1080 representative set of results. The scale bars correspond to 10 µm. Error bars indicate SD  
1081 determined from triplicate samples. \**p* < 0.05, \*\**p* < 0.005.

1082 **FIG 4** Binding and blocking of BNeV VLPs to synthetic HBGAs. (A) Ninety-six-well plates  
1083 were coated with 50 µg/ml BNeV VLPs, GST-tagged-VP8\* protein and GST-tagged P  
1084 particles (10 µg/ml), supernatant of wild-type baculovirus-infected Sf9 cells lysate and GST  
1085 and then incubated with each of the synthetic HBGAs (10 µg/ml). The binding of each  
1086 HBGA to target viral proteins and the control was determined by addition of horseradish  
1087 peroxidase-conjugated streptavidin as described in the Materials and Methods section. (B)  
1088 Alpha1,2-linked-fucose, α1,3/4-linked-fucose, αGal, and GalNAc epitopes were removed  
1089 from each of synthetic HBGAs coted in each well using the corresponding enzyme. After  
1090 incubation of BNeV VLPs at 50 µg/ml, the binding of BNeV VLPs was determined using  
1091 hyperimmune serum against BNeV capsid protein, followed by addition of horseradish  
1092 peroxidase-conjugated goat anti-rabbit IgG antibody. The signal intensities for Figures A and  
1093 B graphs were visualized using the TMB at 450 nm in three independent experiments. Error  
1094 bars indicate SD from triplicate samples.

1095 **FIG 5** Blocking of the binding of GST-VP8\* protein of bovine rotavirus P[5] WC3 strain to  
1096 synthetic HBGAs. Alpha1,2-linked-fucose, α1,3/4-linked-fucose, αGal, and GalNAc epitopes

1097 were removed from each synthetic HBGA using the corresponding enzyme. Reduction in  
1098 binding specificity to the VP8\* protein of the bovine rotavirus P[5] WC3 strain was  
1099 determined using the HBGA-binding assay. The signal intensities were visualized by TMB at  
1100 450 nm in three independent experiments. Error bars indicate SD from triplicate samples.

1101 **FIG 6** Binding between BNeV VLPs and saliva samples. (A) Expression levels of each  
1102 HBGA in eight boiled bovine saliva samples coated onto 96-well plates were determined  
1103 using mouse monoclonal antibodies specific for each HBGA, followed by the addition of  
1104 horseradish peroxidase-conjugated goat anti-mouse IgG and IgM antibody. After  
1105 characterization of HBGA expression levels in each saliva sample, binding specificity of  
1106 BNeV VLPs and VP8\* protein of bovine rotavirus strain WC3 to each saliva sample was  
1107 determined by saliva-binding assay. The horizontal red line represents the OD values for  
1108 BNeV VLP binding for each saliva sample. The horizontal blue line represents the OD values  
1109 for VP8\* protein of bovine rotavirus WC3 strain. (B) Expression levels of each HBGA in 53  
1110 human saliva samples were determined as described above. Binding specificity of BNeV  
1111 VLPs to each saliva sample was determined by saliva-binding assay as described in the  
1112 Materials and Methods section. The horizontal red line represents the OD values for BNeV  
1113 VLP binding for each saliva sample. Binding of BNeV VLPs to each sample was visualized  
1114 using TMB and measured at 450 nm in three independent experiments. Error bars indicate  
1115 SD from triplicate samples.

1116 **FIG 7** Binding of the rotavirus VP8\* protein and human NoV P particles to human saliva  
1117 samples. (A) The GST-VP8\* protein of the human rotavirus P[25] Dhaka6 strain was tested  
1118 as a positive control for binding to a panel of saliva samples from 53 human individuals.  
1119 Binding results for the individual saliva samples were sorted by A type and non-A type



1120 HBGA signals for the individual saliva samples. (B) The GST-P particles of the human  
1121 norovirus strain VA387 (GII.4) were tested as a positive control for binding to a panel of  
1122 saliva samples from 53 human individuals. The binding of P particles was plotted by sorting  
1123 of the A and B type signals from individual saliva samples. Binding of BNeV VLPs to each  
1124 sample was visualized using TMB and measured at 450 nm in three independent experiments.  
1125 Error bars indicate SD from triplicate samples.

1126 **FIG 8** Blocking of binding of BNeV VLPs and VP8\* protein of bovine P[5] strain WC3 to  
1127 bovine and human saliva samples. (A) Four selected bovine saliva samples expressing either  
1128 A type (H+/A+/B-) or H type (H+/A-/B-) were coated onto 96-well plates prior to removal  
1129 of  $\alpha$ 1,2-linked-fucose,  $\alpha$ 1,3/4-linked-fucose,  $\alpha$ Gal, and GalNAc epitopes from HBGAs by  
1130 pretreatment with a single specific enzyme or a combination of enzymes. A reduction in the  
1131 HBGA binding specificity of BNeV VLPs to each bovine saliva sample was determined by  
1132 saliva-binding assay using hyperimmune serum against BNeV capsid protein as described in  
1133 the Materials and Methods section. (B) The GST-VP8\* protein of the bovine rotavirus P[5]  
1134 WC3 strain was used as a positive control. Four bovine saliva samples expressing either A-  
1135 type (H+/A+/B-) or H-type (H+/A-/B-) HBGAs were used to remove  $\alpha$ 1,2-linked-fucose,  
1136  $\alpha$ 1,3/4-linked-fucose,  $\alpha$ Gal, and GalNAc epitopes from HBGAs in the saliva samples by  
1137 individual or combinatorial enzyme pretreatment. A reduction in the HBGA binding  
1138 specificity of VP8\* protein of bovine rotavirus strain WC3 was determined by a saliva-  
1139 binding assay using hyperimmune serum against VP8\* protein. (C) Six human saliva samples  
1140 expressing either A type (H+/A+/B-) or H type (H+/A-/B-) were used to remove  $\alpha$ 1,2-  
1141 linked-fucose,  $\alpha$ 1,3/4-linked-fucose,  $\alpha$ Gal, and GalNAc epitopes from HBGAs in the saliva  
1142 samples by pretreatment with each specific enzyme individually or in various combinations.

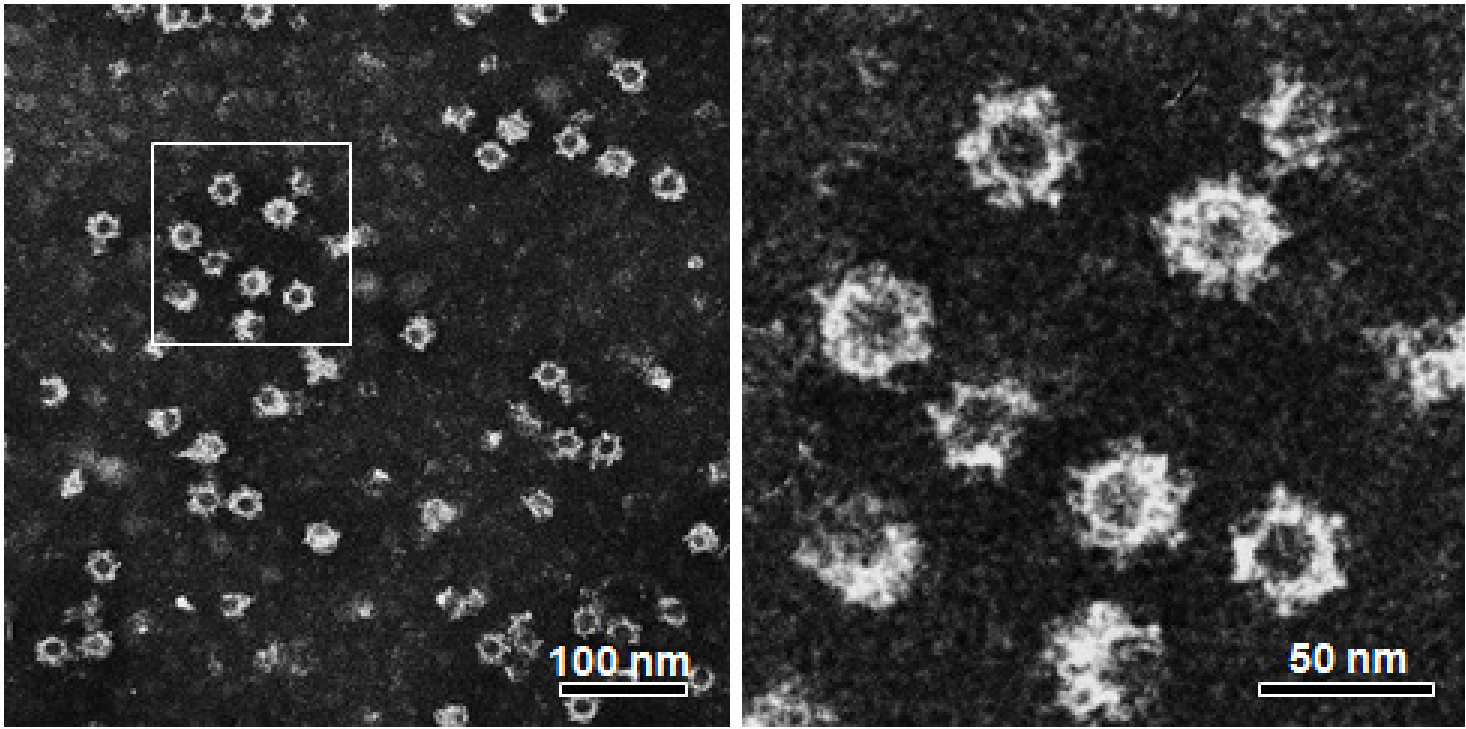
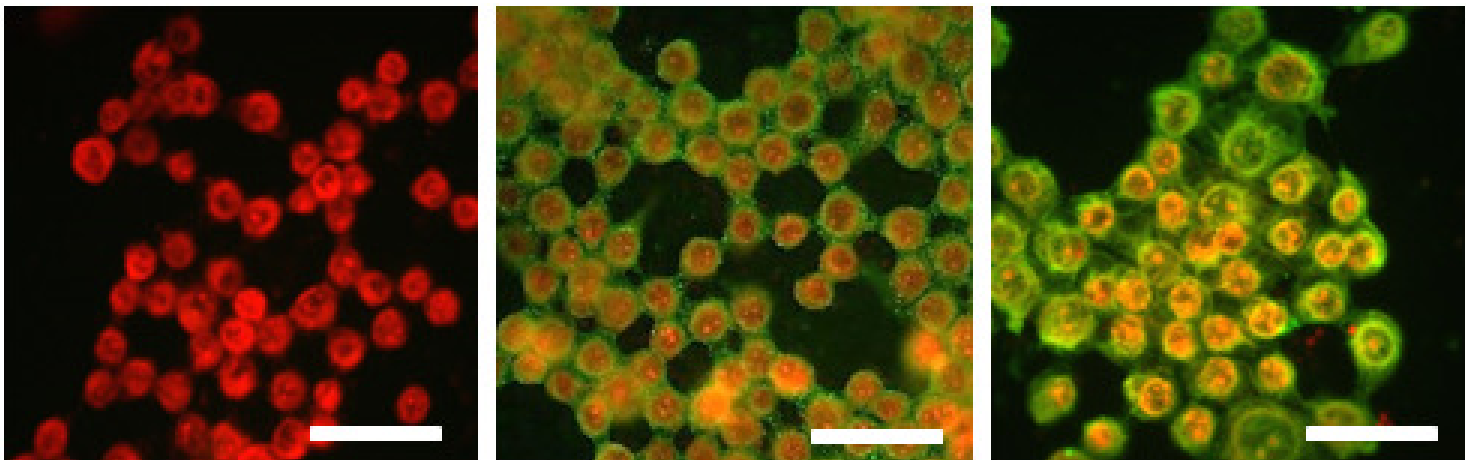
1143 Reduction in the HBGA binding specificity of BNeV VLPs was determined by a saliva-  
1144 binding assay using hyperimmune serum against BNeV capsid protein as described in the  
1145 Materials and Methods section. Blocking of BNeV VLPs to each sample was visualized using  
1146 TMB and measured at 450 nm in three independent experiments. Error bars indicate SD from  
1147 triplicate samples.

1148 **FIG 9** Determination of BNeV-binding HBGA epitopes in MDBK and Caco-2 cells. (A)  
1149 Bovine kidney epithelial MDBK cells were pretreated with or without each specific enzyme  
1150 ( $\alpha$ 1,2-L-fucosidase,  $\alpha$ 1,3/4-L-fucosidase,  $\alpha$ -galactosidase, or  $\alpha$ N-acetylgalactosaminidase)  
1151 individually or in various combinations as indicated, mock-treated or applied with 10  $\mu$ g/ml  
1152 AF594-labeled BNeV VLPs, and then examined by confocal microscopy. (B) To remove  
1153  $\alpha$ 1,2-linked-fucose,  $\alpha$ 1,3/4-linked-fucose,  $\alpha$ Gal, GalNAc epitopes, or  $\alpha$ 1,2- and  $\alpha$ 1,3/4-  
1154 linked-fucoses together, human colorectal adenocarcinoma Caco-2 cells were pretreated with  
1155 or without each individual enzyme or combinations of the enzymes as indicated, mock-  
1156 treated or applied with AF594-labeled BNeV VLPs at 10  $\mu$ g/ml, and then examined by  
1157 confocal microscopy. All experiments were performed three independent times, and Figures  
1158 A-C show one representative set of results. The scale bars correspond to 50  $\mu$ m.

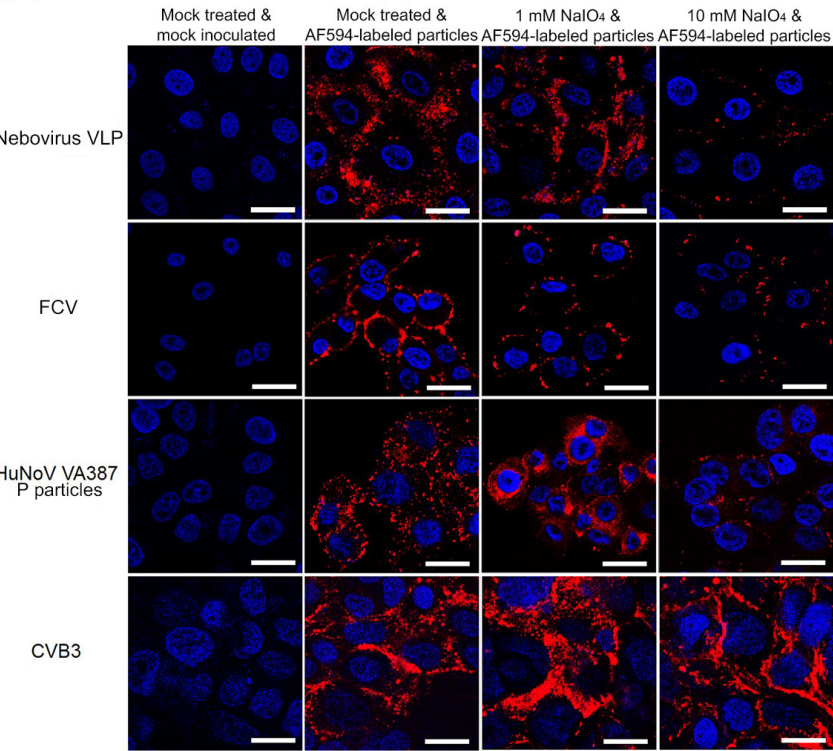
1159 **FIG 10** Binding of BNeV VLPs to parent and transfectant CHO cells stably expressing  
1160 HBGAs. (A) The expression of target HBGA in the parental CHO (H<sup>-</sup>/A<sup>-</sup>/B<sup>-</sup>) cells, single-  
1161 transfectant CHO cells expressing the H antigen (H<sup>+</sup>/A<sup>-</sup>/B<sup>-</sup>) or the  $\alpha$ Gal antigen (H<sup>-</sup>/A<sup>-</sup>/B<sup>-</sup>/  
1162 / $\alpha$ Gal<sup>+</sup>), and double-transfectant CHO cells expressing either the A antigen (H<sup>+</sup>/A<sup>+</sup>/B<sup>-</sup>) or  
1163 the B antigen (H<sup>+</sup>/A<sup>-</sup>/B<sup>+</sup>) were determined using antibodies specific for each target HBGA  
1164 via confocal microscopy. (B) The parental and transfectant CHO cells stably expressing  
1165 target HBGA were applied with AF594-labeled BNeV VLPs (10  $\mu$ g/ml), and then examined

1166 by confocal microscopy. All experiments were performed three independent times, and each  
1167 panel shows one representative set of results. The scale bars correspond to 50  $\mu\text{m}$ .

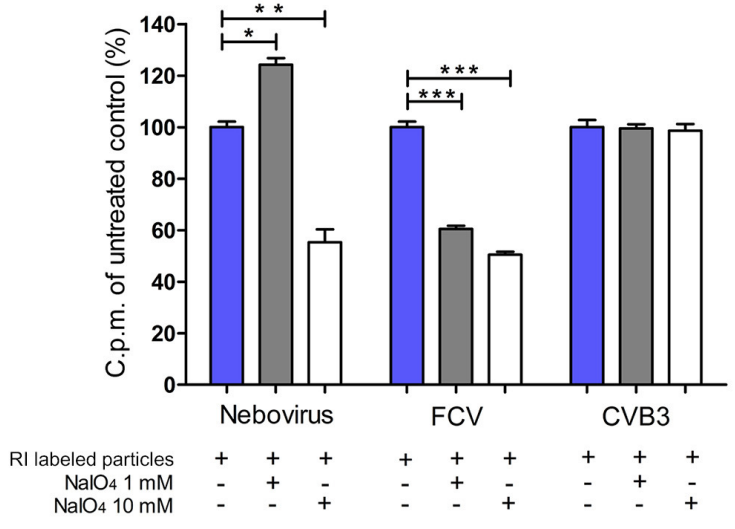
1168 **FIG 11** Binding inhibition of BNeV VLPs to bovine small intestinal epithelium by HBGA  
1169 epitope-specific enzymes. (A) H<sup>+</sup>/A<sup>+</sup>/B<sup>-</sup>/Le<sup>y</sup><sup>+</sup>/ $\alpha$ -Gal<sup>+</sup> tissue sections and (B) H<sup>+</sup>/A<sup>-</sup>/B<sup>-</sup>  
1170 /Le<sup>y</sup><sup>+</sup>/ $\alpha$ -Gal<sup>+</sup> tissue sections were pretreated with enzymes specifically cleaving  $\alpha$ -1,2-linked-  
1171 fucose,  $\alpha$ -1,3/4-linked-fucose,  $\alpha$ Gal, or GalNAc prior to incubation with BNeV VLPs at 10  
1172  $\mu\text{g}/\text{ml}$ . Binding of BNeV VLPs was then detected using a rabbit hyperimmune serum against  
1173 BNeV capsid protein. The experiment was performed in triplicate and one representative set  
1174 of results is shown. The scale bars correspond to 200  $\mu\text{m}$ .

**A****B**

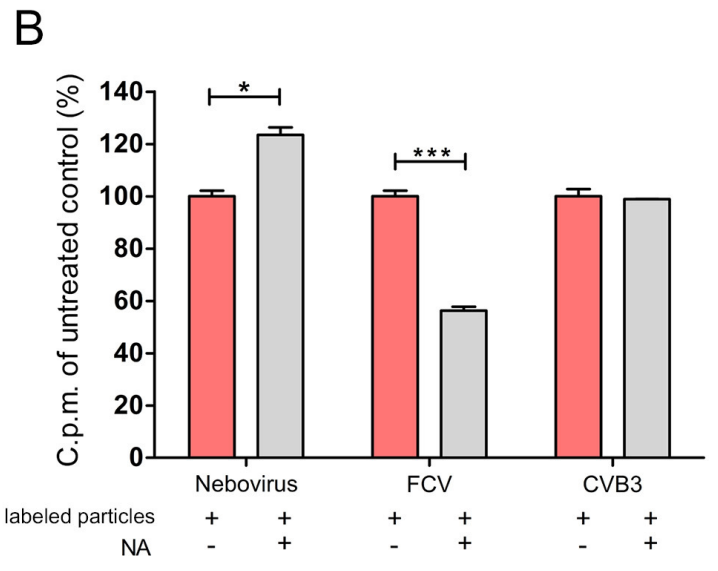
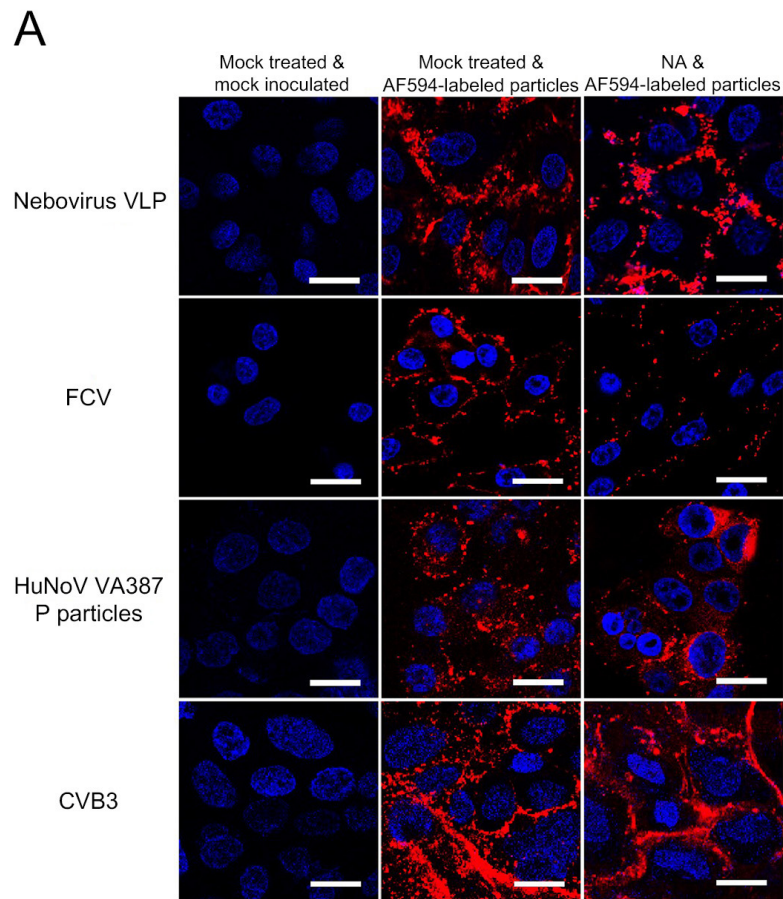
A



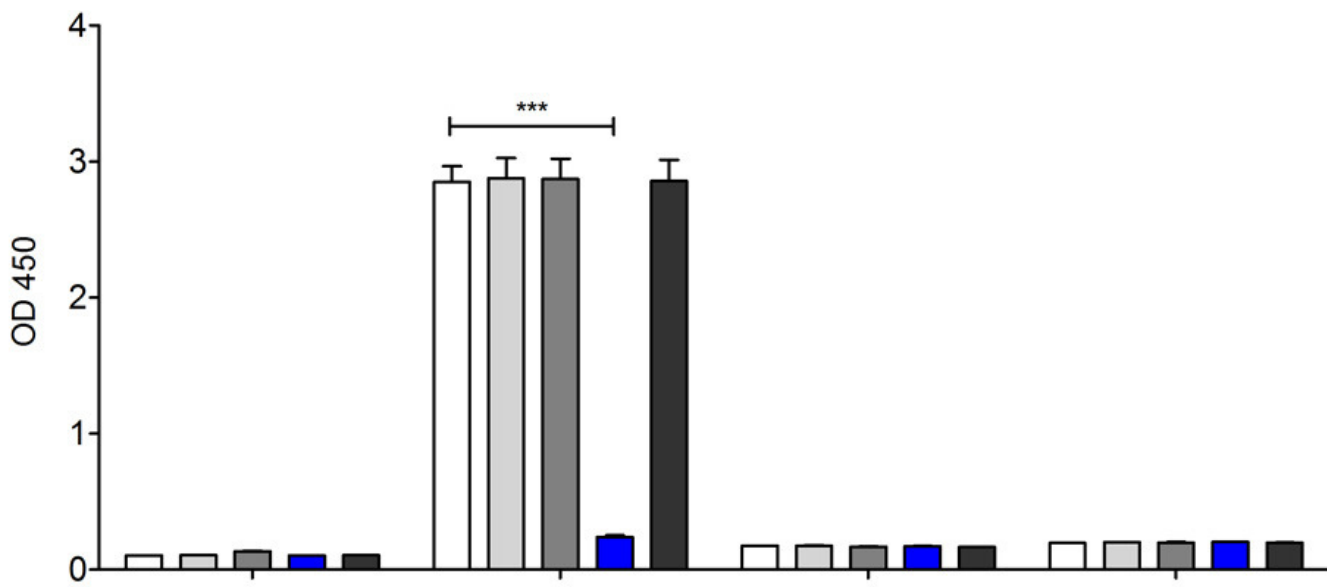
B



RI labeled particles	+	+	+	+	+	+	+	+
NaIO4 1 mM	-	+	-	-	+	-	+	-
NaIO4 10 mM	-	-	+	-	-	+	-	+



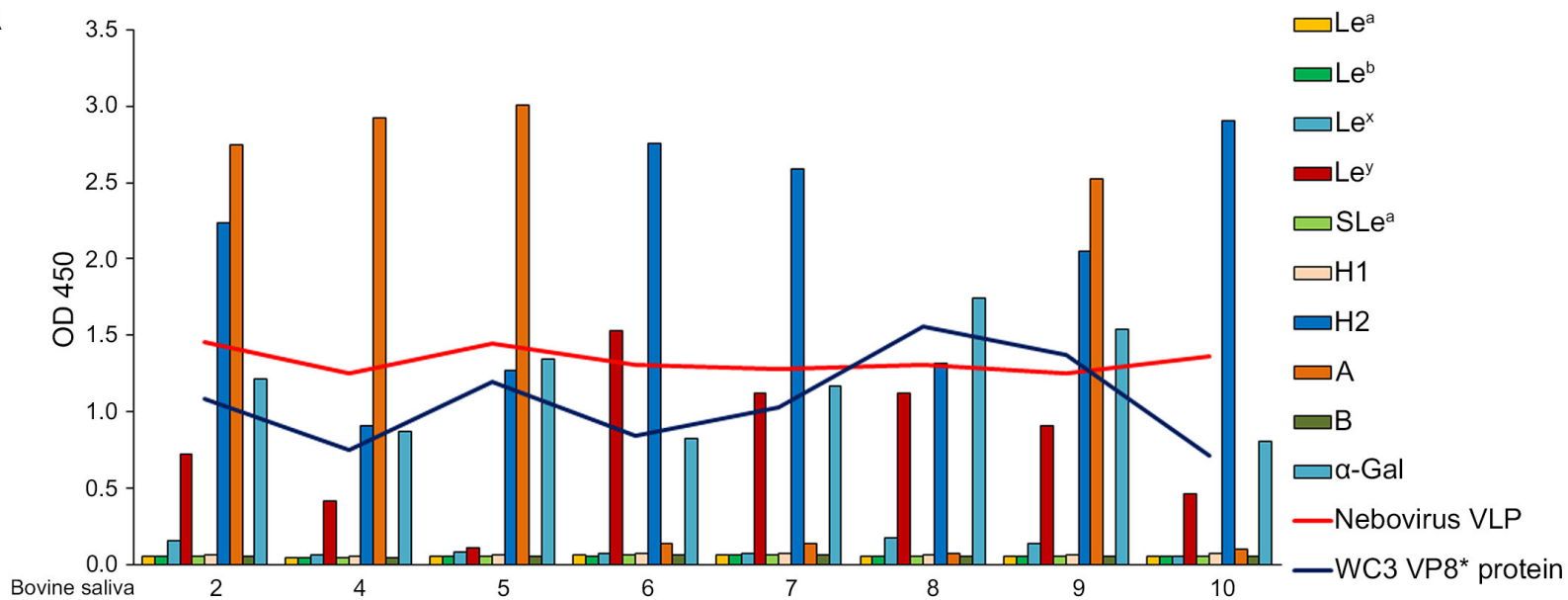




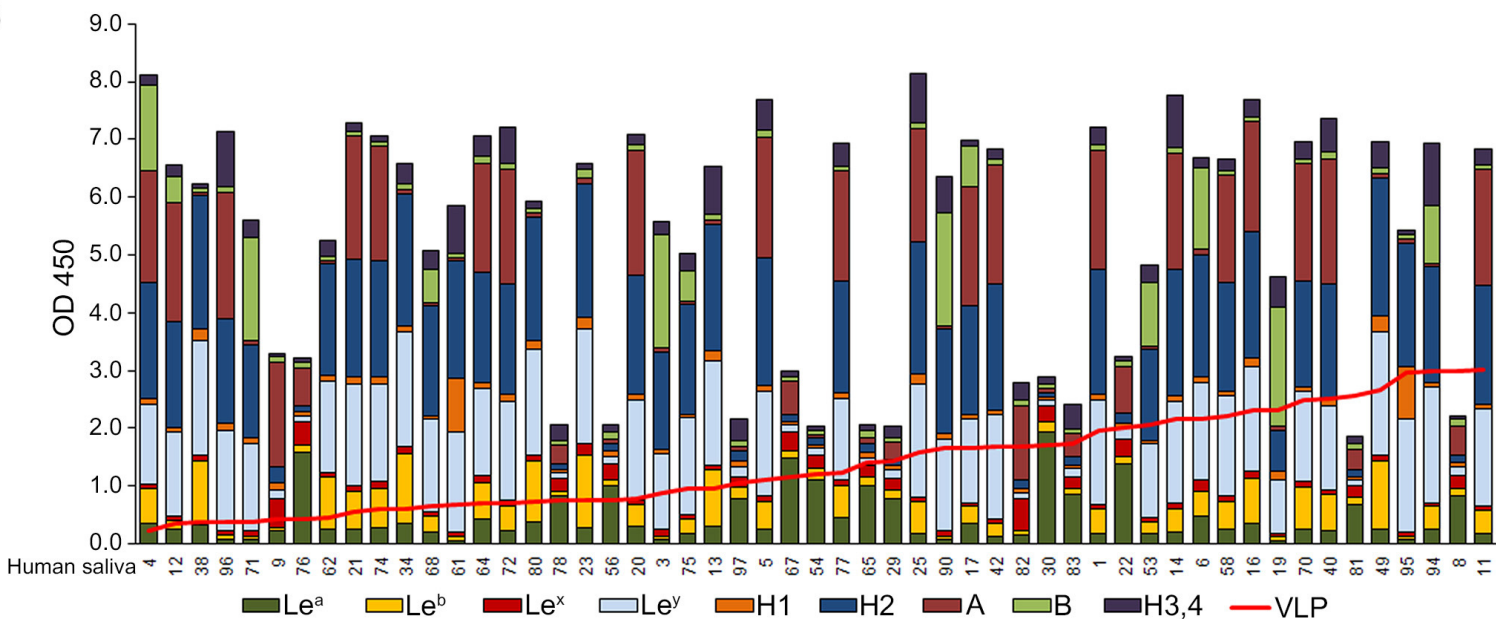
HBGAs	H					α-Gal					A					B				
VP8* of rotavirus WC3 strain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α1,2-fucosidase	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-
α1,3/4-fucosidase	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-
α-galactosidase	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-
αN-acetylgalactosaminidase	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+

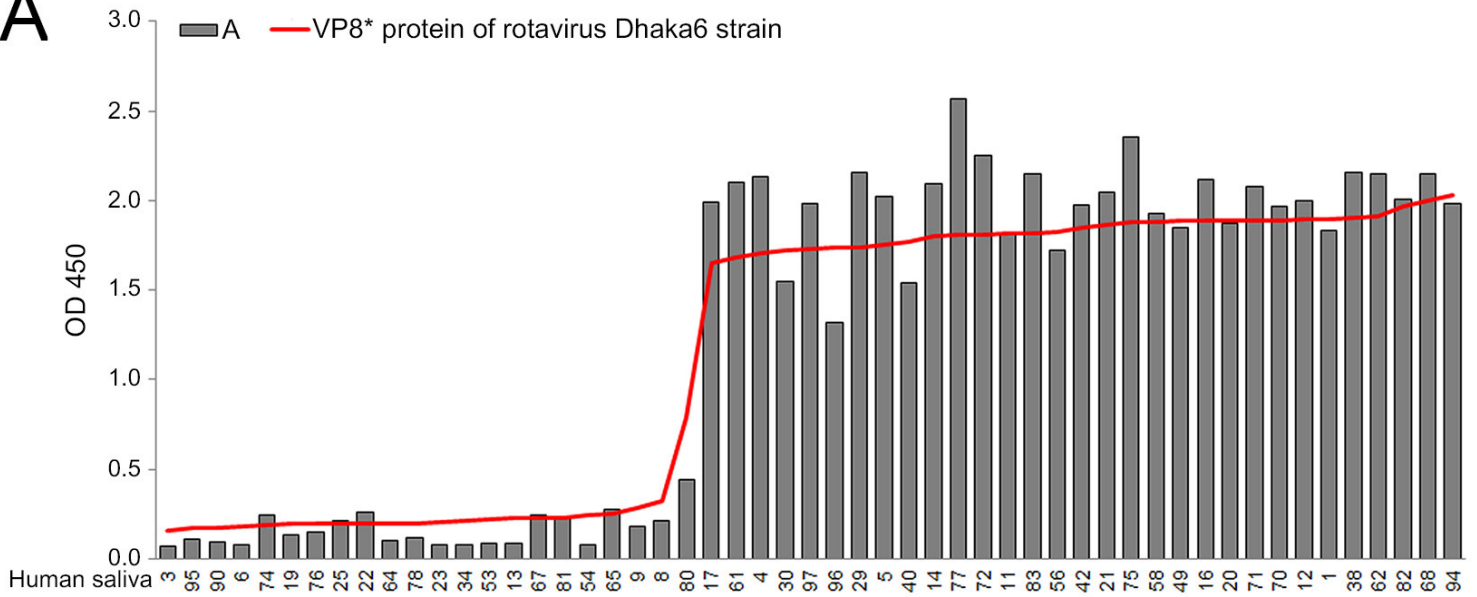
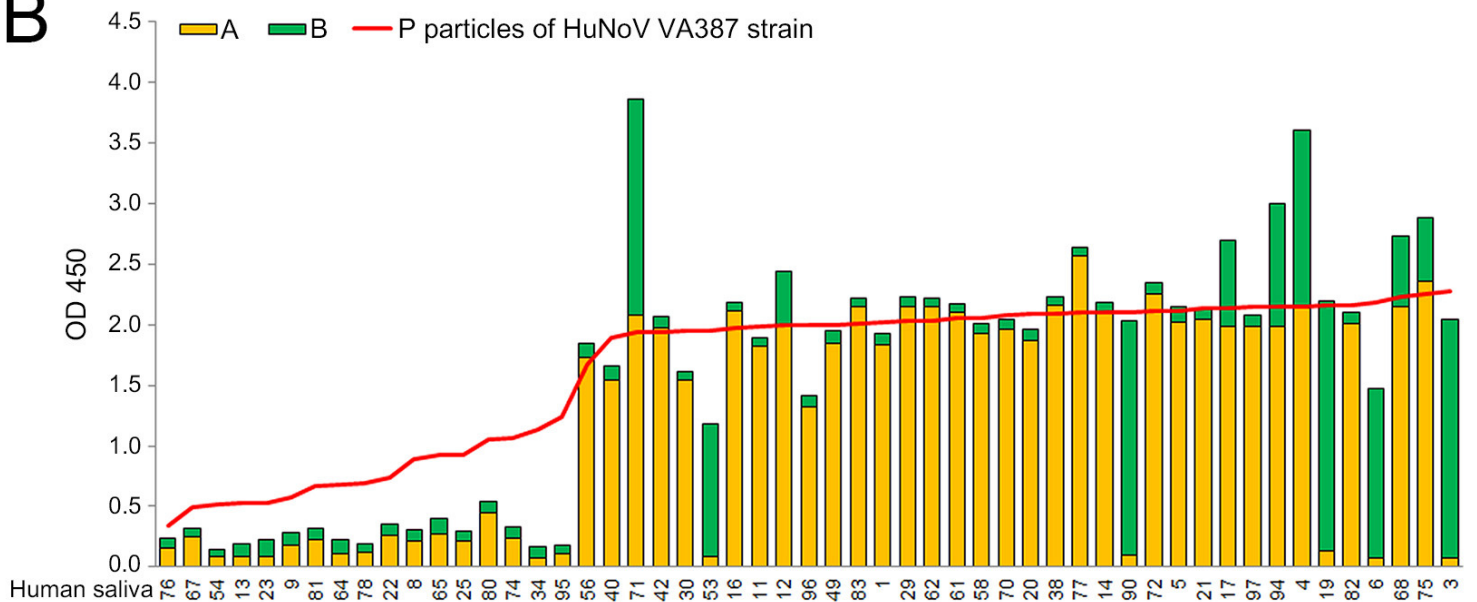


A

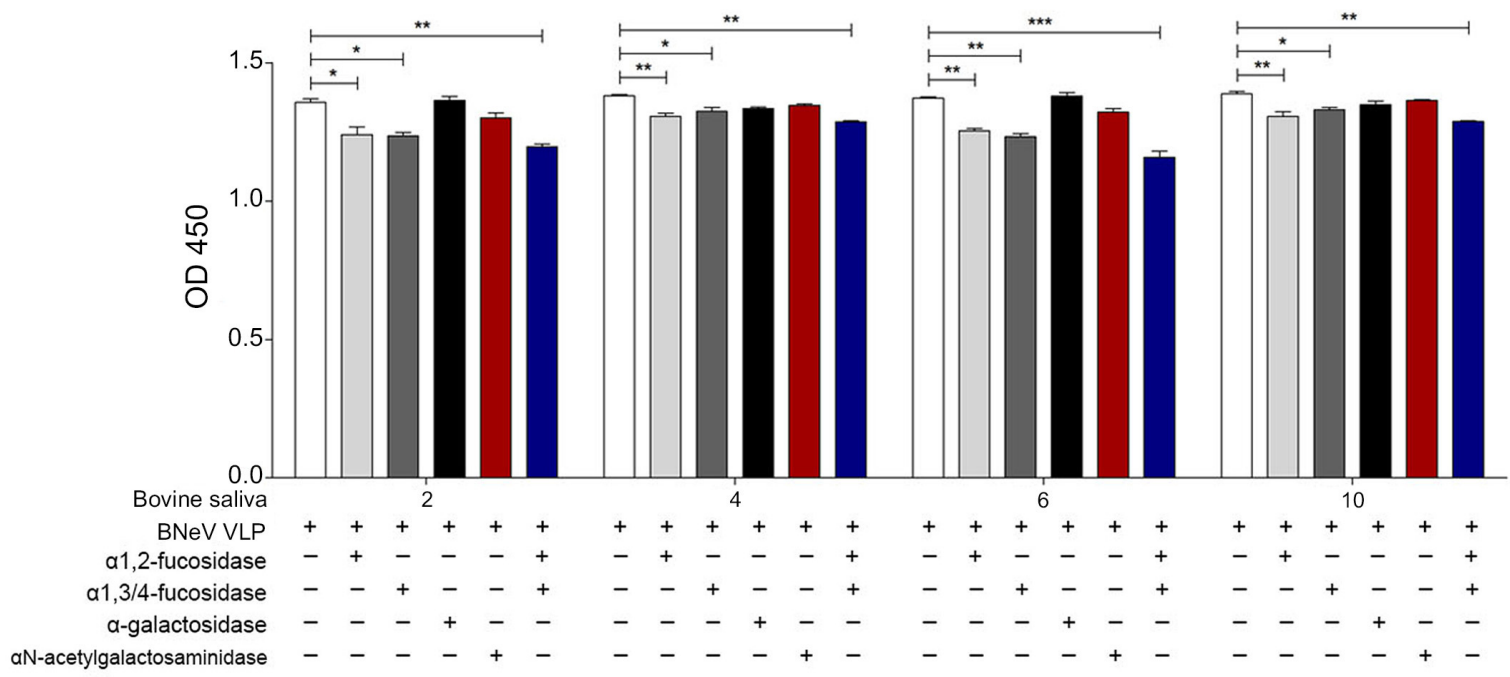


B

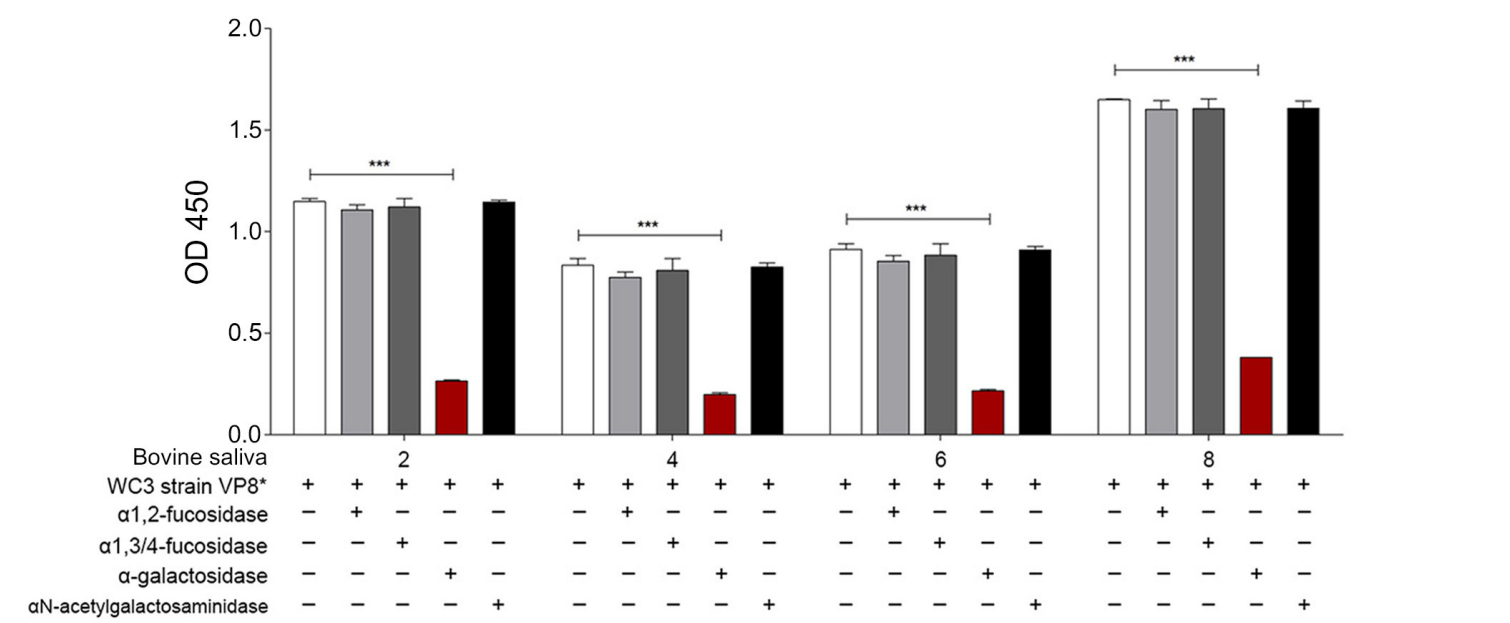


**A****B**

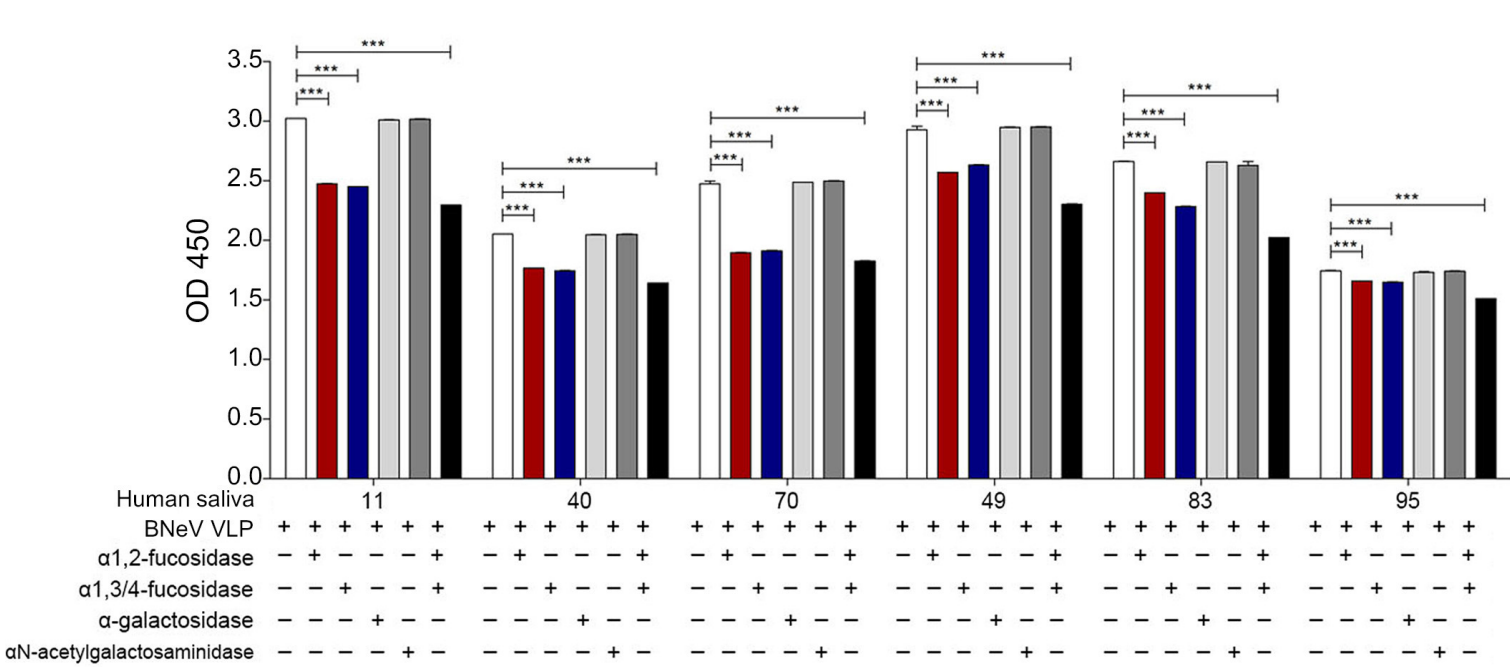
**A**



**B**

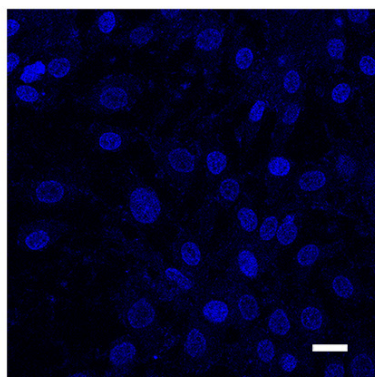
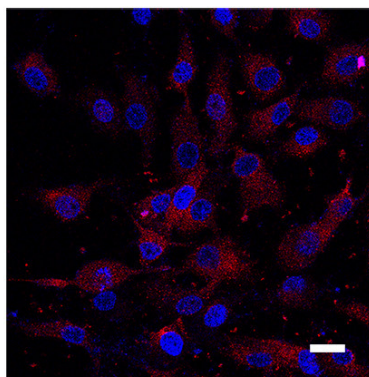
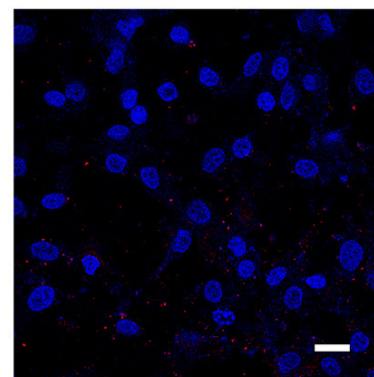
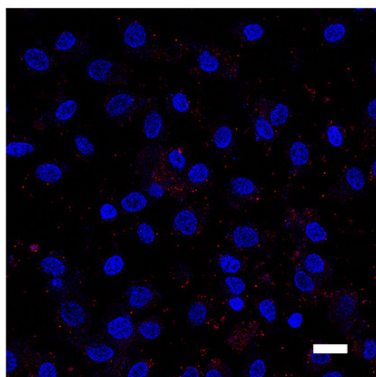
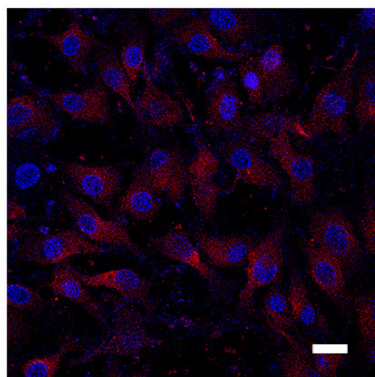
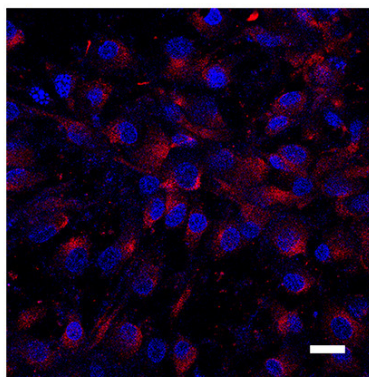
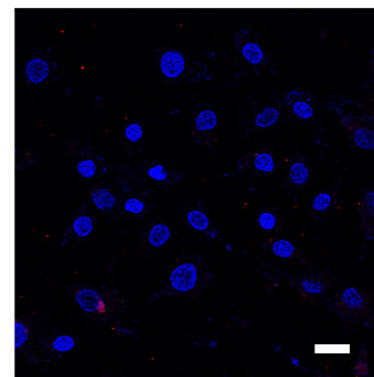


**C**

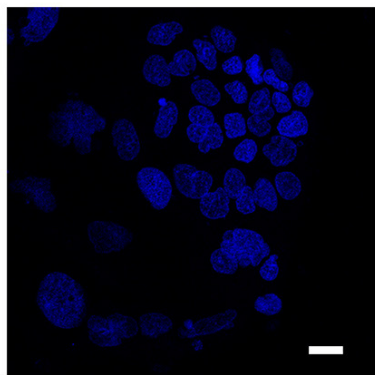
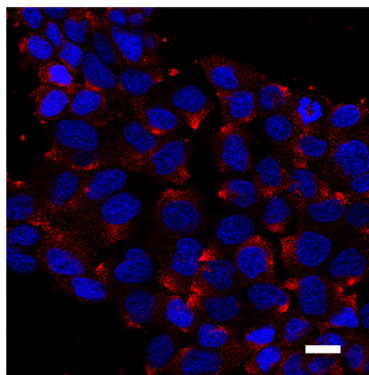
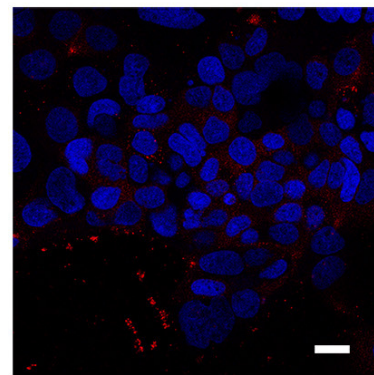
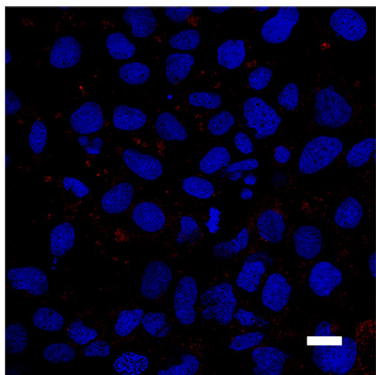
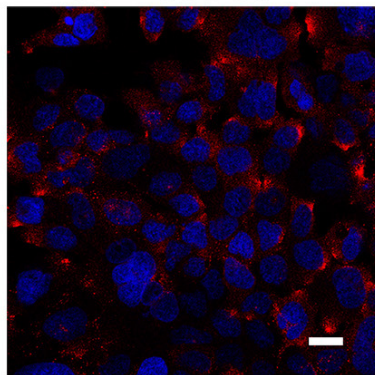
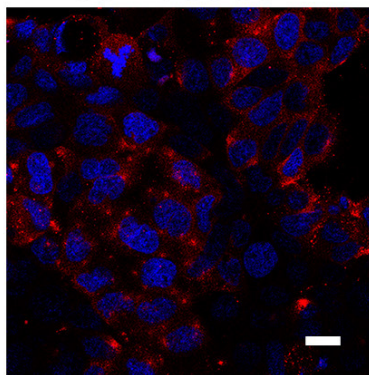
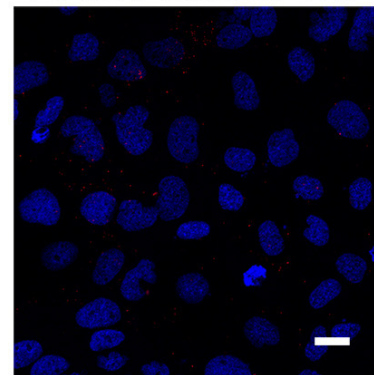


**A**

MDBK cells

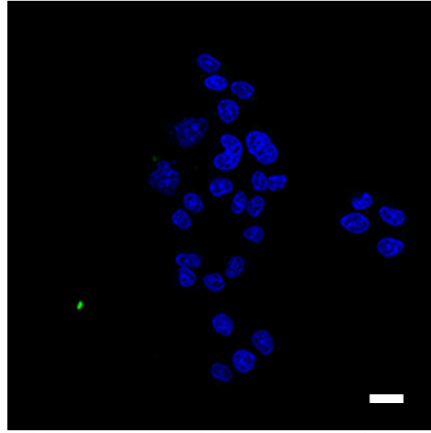
Mock treated &  
mock inoculatedMock treated &  
AF594-labeled VLP $\alpha$ 1,2-fucosidase &  
AF594-labeled VLP $\alpha$ 1,3/4-fucosidase &  
AF594-labeled VLP $\alpha$ -galactosidase &  
AF594-labeled VLP $\alpha$ N-acetylgalactosaminidase &  
AF594-labeled VLP $\alpha$ 1,2- +  $\alpha$ 1,3/4-fucosidase &  
AF594-labeled VLP**B**

Caco-2 cells

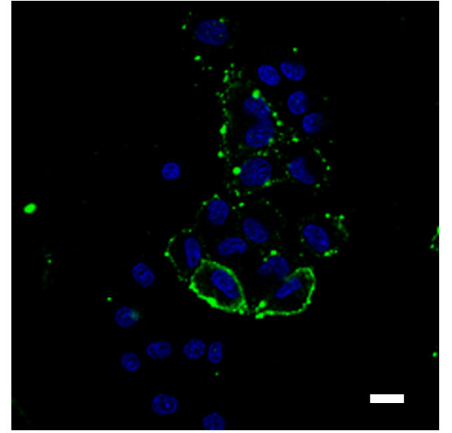
Mock treated &  
mock inoculatedMock treated &  
AF594-labeled VLP $\alpha$ 1,2-fucosidase &  
AF594-labeled VLP $\alpha$ 1,3/4-fucosidase &  
AF594-labeled VLP $\alpha$ -galactosidase &  
AF594-labeled VLP $\alpha$ N-acetylgalactosaminidase &  
AF594-labeled VLP $\alpha$ 1,2- +  $\alpha$ 1,3/4-fucosidase &  
AF594-labeled VLP

A

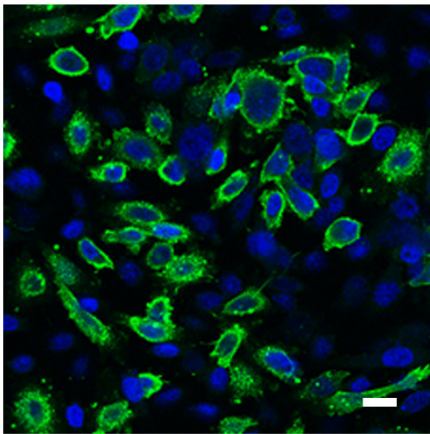
CHO-K1



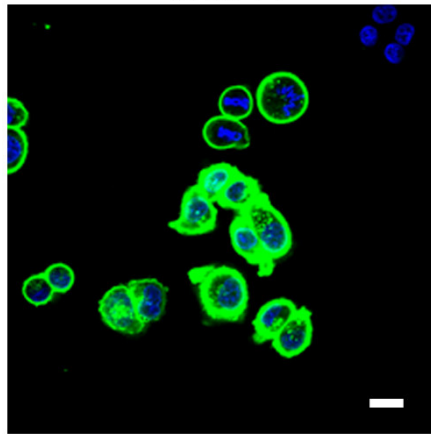
CHO H



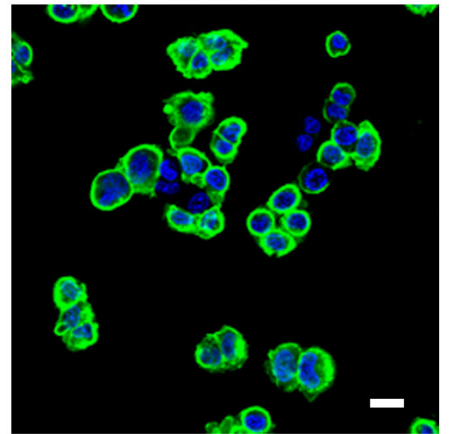
CHO  $\alpha$ -Gal



CHO A

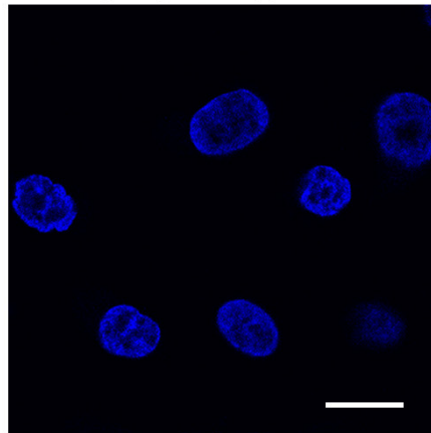


CHO B

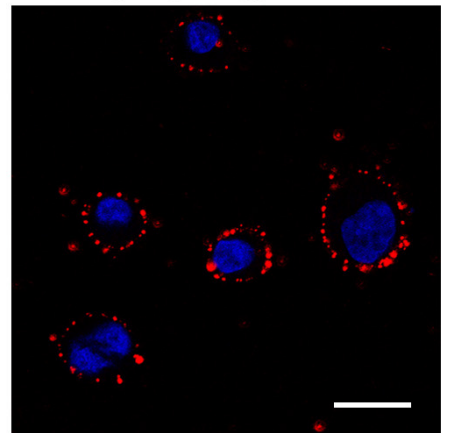


B

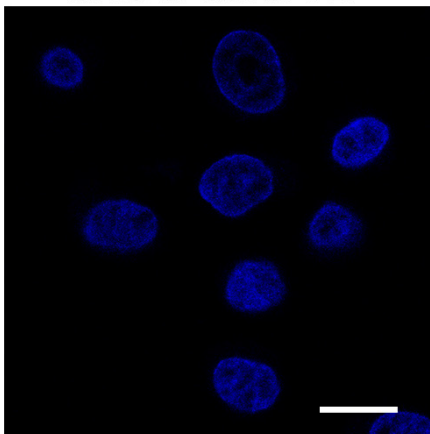
CHO-K1 & VLP



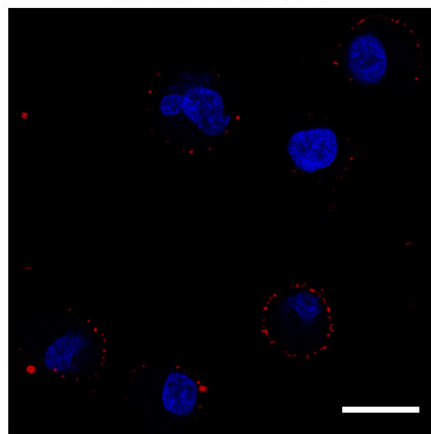
CHO H & VLP



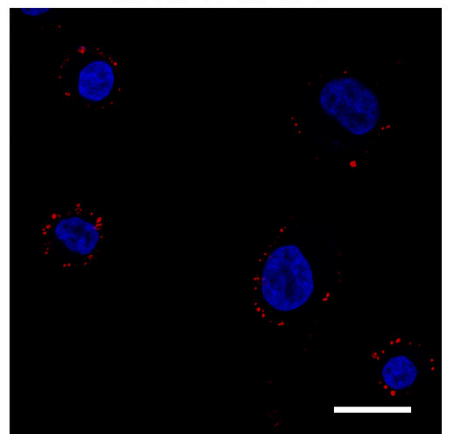
CHO  $\alpha$ -Gal & VLP

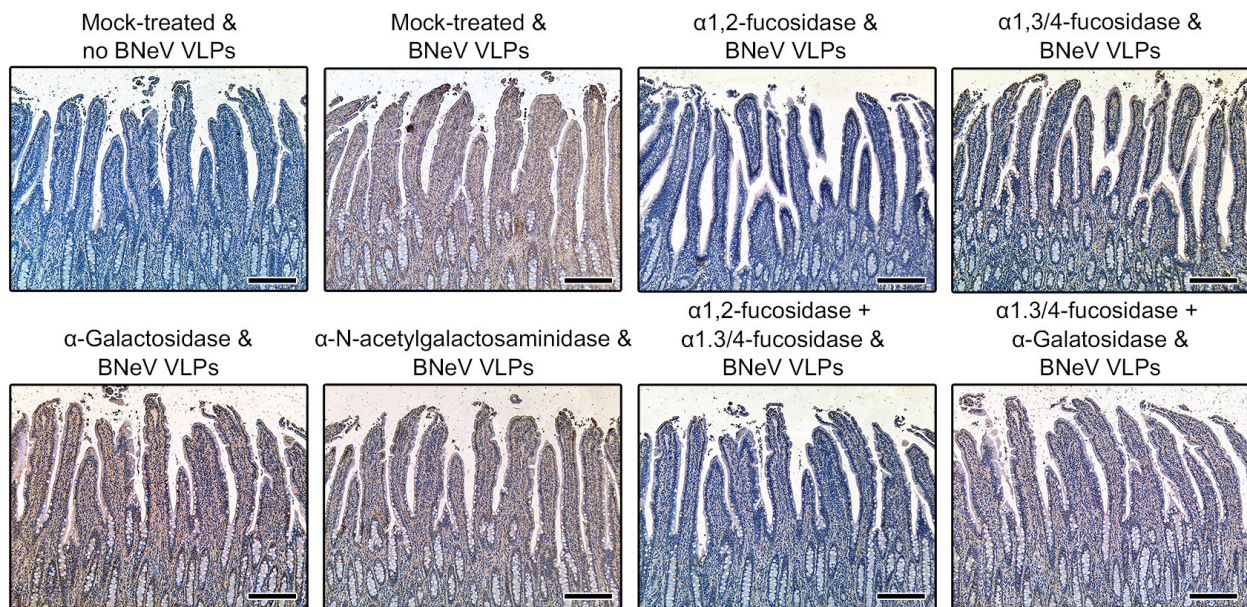


CHO A & VLP



CHO B & VLP



**A****(H+/A+/B-)****B****(H+/A-/B-)**